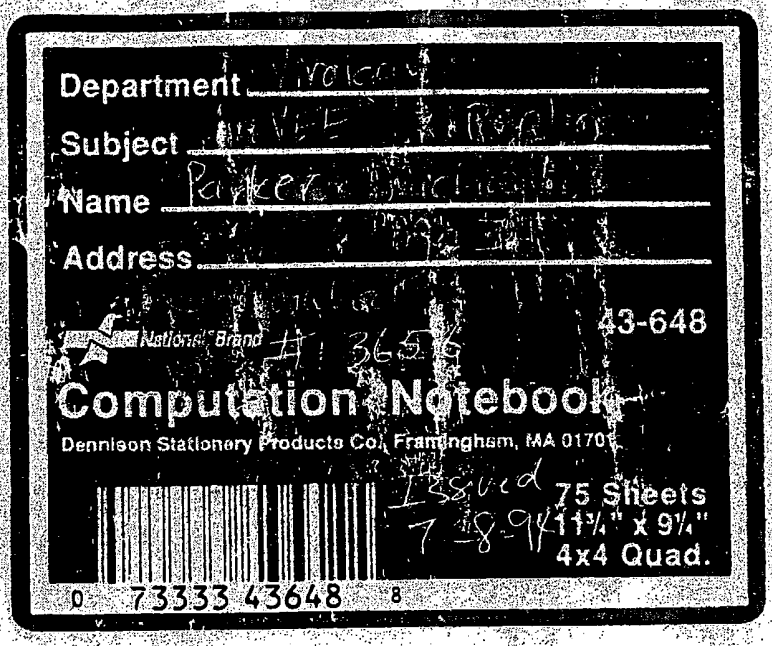
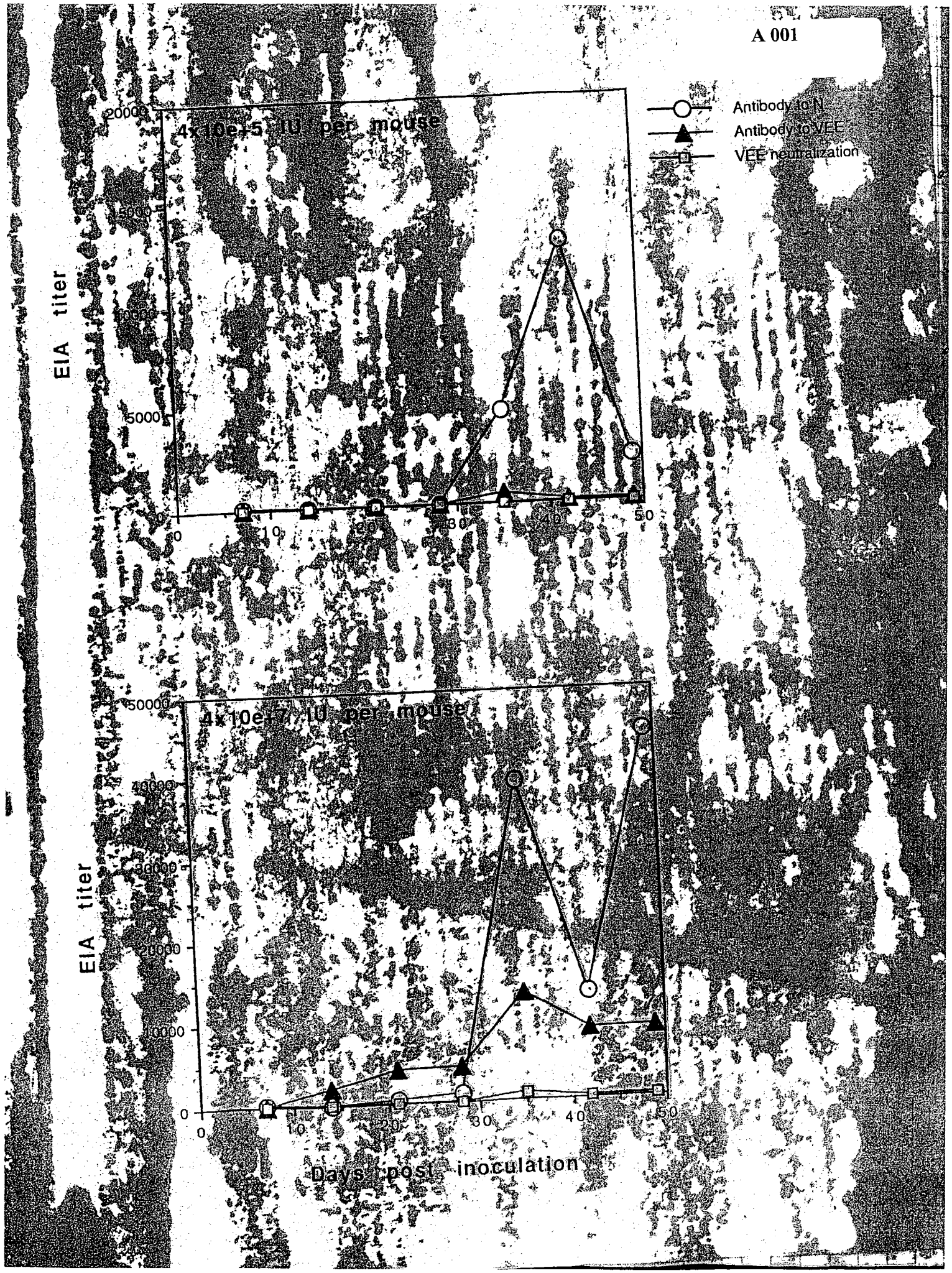

Department
Subject
Name
Address
National Brand
43-648
Computation Notebook
Dennison Stationery Products Co., Framingham, MA 01701
Issued
7-18-94
75 Sheets
11¾" x 9¼"
4x4 Quad.
0 73333 43648 8

EXHIBIT A

A 001


4x10e+5 IU per mouse
EIA titer
20000
15000
10000
5000
0
0
10
20
30
40
50
Antibody to N
Antibody to VEE
VEE neutralization
4x10e+7 IU per mouse
EIA titer
50000
40000
30000
20000
10000
0
0
10
20
30
40
50
Days post inoculation

BEST AVAILABLE COPY

7/28/1994

Transformation

BRL MAX Efficiency DH5α™ Competent Cells

1. 10pg of DNA (U3000) for 50µl BRL DH5α Cells
2. Keep DNA and Cells on ice (to thaw cells)
3. Put 50µl cells into µ tubes containing 10 ng of U3
4. Incubate on ice for 30 min
5. Heat shock 45 seconds in 42°C, no shaking
6. Ice 2 min
7. add 1 ml of SOC (pre-heat to 37°C)
8. Shake at 37°C for 1 hour
9. Spreadd 100µl of this reaction and 900µl (centrifuge) on LB plates containing 100 µg/ml Amp.

3000 (1) DNA VE 1A strain 272-3 (1.1mg/ml stock) 10µl containg 10ng (1 µg/
(2) DNA 10µl -"- 100ng (10ng/
(3) DNA 10µl -"- 1µg (100ng/

2x 3ml overnight cultures A 3014

Cleavage pU3000 ApaI + RspII

	Water	30µl
1.1µg/µl	DNA	3.2µl
	BRL REact (5)	3.6µl
20u/µl	Rsp II	0.5µl
10u/µl	Apa I	1.2µl
		38.5µl → 1hr at 37°C

+ 60µl of water
+ 100µl of phenol-chloroform
1 min of vortex
upper phase + 10µl 1M NaCl + 300µl 95% EtOH
-70°C o/n

4/2/1998 pV3000 cleaved w/ RsrII & AflaI & precipitated o/n at -20°C
Friday Spin down 15 min wash 1x 75 EtOH (no pellet)
dryed at the bench ↓ Resp.
dissolve in 45 µl water total stored
(final 500µM) add 0.5 µl dNTP (50mM) in Eppi. Poly
5 µl 10 x T4 (P) Polymerase
1 µl T4 (P) Gibco BRL 2.4 U/µl dried precipitate
big pellet
washed 2x
with EtOH

pt 20 min

+ 50 µl water
+ 100 µl phenol
+ 10 µl of 1M NaCl
+ 300 µl EtOH → -20°C 20'
+ 0.5 µl tRNA

Spin down, wash twice w/ 75% EtOH.
dry on the bench.

dissolve in 30 µl of water
10 µl load on the 1% agarose gel

Results of the transformation of E. coli w/ pV3000

for 50 µl of BRL Max Efficiency Comp. cells (opt 100)

① 10 ng DNA (1 ng/µl) 10 µl total DNA-sol. / 100 µl per plate (400 ea
② 100 ng DNA (10 ng/µl) — / — / — (540
③ 1 µg DNA (100 ng/µl) — / — / — (57 40a
per plat

Results of 1% Agaros are interpreted as follows)

① λ Hindiii marker
② pV3000 800 ng uncleaved
③ pV3000 (AflaI & RsrII / T4 DNA pol)
④ pV3000 supernatant (just to check precipitation efficiency)
⑤ 1Kb marker

Two bands in lane ③ correspond probably to linearized pV3000 (upper) and 6.5 Kb and 7.0 Kb (smaller). Concentration ~ 5 ng/10 µl ⇒ 0.5 ÷ 1 ng/µl

Ligation reaction: P1

DNA pV3000 as lane ③ on the gel (p. 2)	15 µl
10x stock (w/ rATP)	2 µl
(2.000 u/µl NEB) T4 DNA ligase	2 µl

10°C overnight

7/30/199

Saturday

1. P1 (20 µl) + 100 µl water + 100 µl phenol, spin 5 min; upper phase + 10 µl 1M NaCl + 1 µl tRNA 10.6 µg/µl + 400 µl 96% EtOH, 20' at [illegible] spin down [illegible] with 1x 75% EtOH, dry (speedvac) dissolve in 7 µl water

7 µl water
1 µl React 1 buffer
0.7 µl EcoR I 10u/µl
0.7 µl Kpn I 10u/µl

37°C 15^00 ÷ 16^00
10' at 65°C

in order to cleave plasmids resulting from ligated linearized forms (Fig. on p. 2)

DNA mini preps Wizard Kit (from pV3000 cells) (as in protocol Promega) no. 1 and 2; no. 3. and 4. pro 1% Agarose / TBE

①	x Hind III	6 µl
②	5 µl ①	~200 ng/µl
③	5 µl ②	pV3000 purified ~200 ng/µl
④	1 kb	6 µl

transformation into 100 µl 7 MAX Efficiency Comp cells

Cleavage pV3000 ApaI + RsR II

DNA 45 µl (~10 µg)
⑤ React 10x 10 µl
Apa 5 µl (20u/µl, restrap) + 2.5 µl (20u/µl, enzyme)
RsR II 2.5 µl opus genetics + 2.5 µl biolab
H2O 35 µl

37°C 16 30 ÷ 18^00

7/31/94
Sunday

P1 colonies

P1 - 10 µl - 0 colonies
P1 - 100 µl - 1 colony
P1 - 100 µl - 3 colony (••)
P1 - 900 µl - 3 colony (• •)

P1 : Σ (7) colonies

o/n at 37 inoculated 3 ml - LB amp

1% Agarose/TBE
① λ Hind III
② 5 µl of pV3000 ApaI/RsrII
③ 1 Kb

8/1/94
Monday

Карточку можно интерпретировать как полное расщепление, но для верности лучше повторить очередную реакцию после T4 DNA pol.

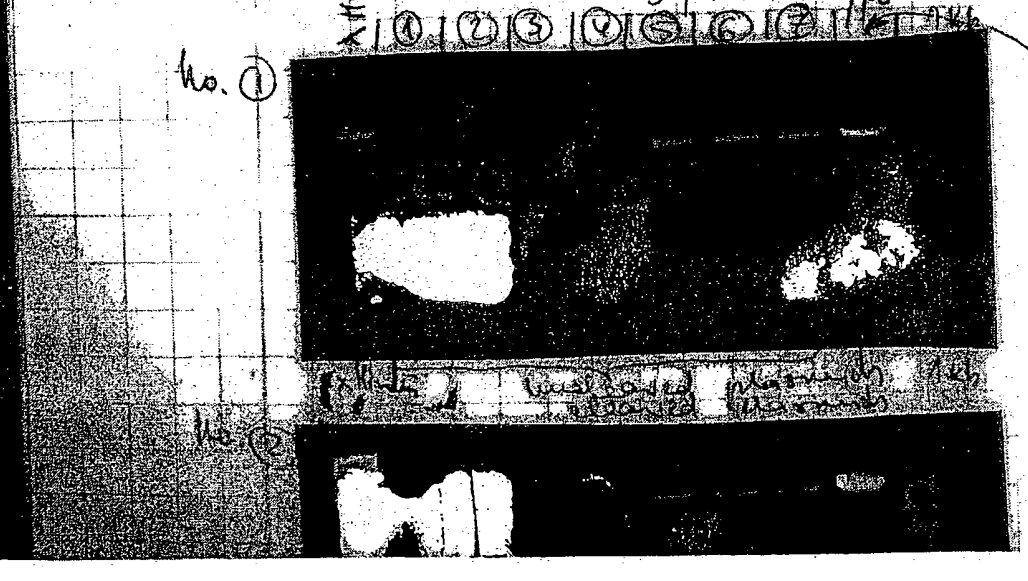
① 1. Mini-preps Nos 1÷7 from P1 clones w/ Wizard mini-prep kit — 5 µl - 1% TBE

2. pV3000 cleaved w/ ApaI + RsrII — phenol treated, +NaCl + tRNA + EtOH 20' at spin down, wash 2x 7 dry dissolve in 30 µl water — 5 µl - 1% TBE

② 3. Cleavage of P1 1÷7 clones

mix 180 µl H2O
20 µl React 2 10x
3 µl 10 u/µl XbaI
3 µl 10 u/µl XbaI

↑ 20 µl each well + 3 µl of DNA
37°C 1 1 30 : 1 : 400



pV3000 ApaI/RsrII 5 µl

Plasmids 1÷4 linearized by XbaI (MW ~ 2403 kb) ⇒ Clones are cleaved by

Cleavage Nos 1 ÷ 7 and pV3000 w ScaI

mix: H2O 180 µl
10x React 2 20 µl
10u/µl ScaI 5 µl

→ 20 µl
3 µl of DNA
37°C 1 hr

7 µl - 1% Agarose/TBE

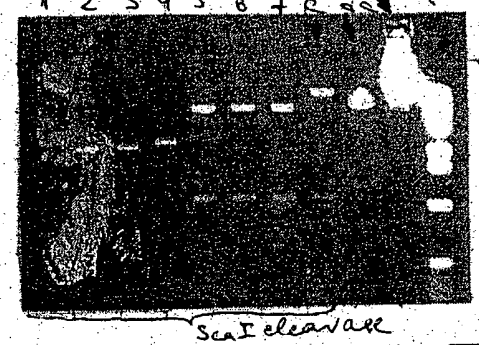
T4 DNA polymerase - mediated reaction

gel No (3)

pV3000 ApaI/BsrII 45 µl (1 µg)
10x T4 (P) buffer 5 µl
100 mM dNTP 0.5 µl
T4 (P) 1 µl

RT 20' + 150 µl water
+ 200 µl phenol-chlor
precipitate w/ 500 µl EtOH
(+ NaCl + tRNA)
20' at -70°C
spin down wash twice 7
dissolve in 20 µl
5 µl - 1% TBE/Agarose

No (3)



ScaI cleavage

P2

DNA 2 µl
H2O 14 µl
10x Buf 2 µl
T4 2 µl
+10°C o/n

Nos 5, 6, 7, pV3000 o/n at 37 w/ SphI (gel No 4)

8/2/9
Tuesd

Transformation of Max Efficiency (MRI) DH5 cells 5 √
① control pV3000/A[redacted]/T4
② P2 (pV3000/ApaI/BsrI/T4/Ligase) [redacted] cells + 20 µ
③ P2 sure (— / — / —) DN

1% Agarose Nos 5 ÷ 7 and pV3000 (SphI)

gel N

cleavage gel (1)

	Sma I	Sca I	Xba I
5	X	X	X
6	X	X	X
7	X	X	X
pV 3000	X	X	X

no cleavage! (or one site)

see also gel no (4) p. 5

gel no (3) p. 5

Xba I (-!)		Sph I OK		Sca I OK		Sma I OK	
pV3000	phelper	pV3000	phelper	pV3000	phelper	pV3000	phelper
9767		11425	4425	12435	5435	7553	
3735	3735	2077	2077	1067	1067	4629	5183
	2767					1319	1319

no cleavage in either pV3000 or phelper !!! (or just one site)

Sph I OK | Xba I | Sma I OK | OK

8/3/94 Wednesday

N₂ 5, 6, 7 and pV3000 cl... w/ NcoI

NcoI	
pV3000	phelper
6451	6500
3910	
2051	
1090	

OK

pV3000 K

XbaI ok

pV3000	phelper
9767	
3735	3735
	2767

SphI OK

pV3000	phelper
11425	4425
2077	2077

ScaI OK

pV3000	phelper
12435	5435
1067	1067

SmaI OK

pV3000	phelper
7553	5183
4621	
1319	1319

no cleavage in either pV3000 or phelper (1 cut just one site)

8/3/94 Wednesday

N2, 5, 6, 7 and pV3000 cl[illegible] w/ NcoI

NcoI

pV3000	phelper
6451	6500
3910	
2051	
1090	

OK

Summary: According to four enzymes (NcoI, ScaI, SmaI) P1-5 = P1-6 = P1-7; these correspond to phelper we are looking for. Mike says that XbaI is missing in one of this plasmid

	100	900
P1	2	~50
P2	~50	~300
P1 sure	~50	~300

plates are stored at 4°C

Kanthy & [P2] transformation → (K)

70 µl per 20 µl ligation mix ← Max-efficiency cells seem to work well.

P1-5 is inoculated into 1 ml of NB and grown for 10 hrs, inoculated into 200 ml of NB and grown o/n for preparative DNA isolation (for transcription of RNA)

8/4/1994 Thursday

1. W3000 inoculated (about 7 colonies) from the plate into 1 ml of NB and grown at 37°C for 4 hrs, inoculated into 200 ml of NB and grown o/n at 37°C

2. Wizard [illegible] preparation kit (Promega) helper.

[illegible] P1-5

8/5/1994 Friday

1. Wizard Maxi [illegible] W3000
2. Cleave P1 [illegible] ~20–120 ng/µl
W3000 [illegible] NotI } preparative amounts
(100 µl in 1.5 ml) P1-5 w NotI } (~1 µg) phenol treatment precipitation wash twice w/ 70% EtOH

8

1. 1 µl of P1-5 cleaved in 20µl by Not I
2. 5µl — " —
3. 15µl — " —
4. 10 µl of purified pV3000 linearized
5. 1 KG

The amount of p1-5
~ 100 ng/µl ⇒ ~ 1 µg / 10 µl ⇒
150 µg total

Assuming the yield of purification of the same vectors, 1.5 µg p1-5 of pV3000 plasmids are cleaved with Not I

100 µl of DNA	150 µl of DNA
150 µl of 10 × React ②	50 µl of 10 × R ③
1250 µl of water	300 µl of water
µl Not I	µl Not I
1500 µl	500 µl

1. 10µl of p1-5/Not I + 3 µl dye } digested DNA
2. 10 µl of pV3000/Not + 3 µl dye
3. 10 µl of p1-5/Not I + 3 µl dye } digested DNA
4. 10 µl of pV3000/Not I + 3 µl dye
5. 1 KG ladder

Resumé:
Cleavage complete
+ 1 vol Isopropanol + NaCl
left -20°C o/n

2/6/1994
Saturday

Precipitates are washed once with 75% EtOH, dryed, dissolved in water and combined, total volume 200 µl, treated with phenol - chloroform + NaCl + EtOH 15' at -70°C, spin down, wash twice, dissolve in 45 µl of water.

TRANSCRIPTION REACTION

250 Units Cat #8050 ambion

Water	10X	ambion 25mM ATP/CTP/GTP	25mM GTP	CAP	^{3}H ATP	DNA	pol	
(6.30µl) 5	3	– 9	3	1	1	5	3	Mike's
~~to~~ 20µl	2	no 2µl 75mM T7 (50mM SP6)	–	1	2	2 (1µg)		Megascript kit
9	3	12	–	1	2	3 (1.5µl)		no cap

6 of Promega's 5X

Ambion: 2130 Woodward Street #200
Austin, Texas 78744-1832
(800)-888-0804 phone

with cap I w/out cap II mG(5')ppp(5')G

1. Larasa LSR 3µl
2. MHelp RSRI 4µl
3. PI-5 1.5µl
4. V3000 1.5µl

polymerase 2.5µl (with cap) + 2.0µl (w/o cap)

into II (w/o Cap) Promega 5X 6µl

37°C (in incubator) 2 hrs → –20°C (done!)

BHK D-29 cells (~90% grown)
discard media
add 5ml Tripsin-EDTA, rinse with,
discard (after 2-5 sec of rinsing).
add 3ml of Tripsin-EDTA, rinse until
cells float (~5 min);
add 5ml of EMEM
dispence into flasks (2ml into
each), incubate +37°C
humidity 99%
CO_2 5%

3/7/1994
Sunday

1. Cells are ~40% confluent
(for transformation is necessary
80-90%) ⇒ grown 1 o/n at 37°C

2. estimation in transcription
[redacted]
by the denaturing gel

1 Lassa replicon/cap
2. RsrI /cap
3. P1-5 /cap
4. pV3000 /cap
5. Lassa /cap
6. RsrI /cap
7. P1-5 /cap
8. pV3000 /cap
9. RNA marker
10. Lassa replicon/cap

8/8/1994
Monday

Promega transcription RiboMAX capped

T7 5x buffer	8.0 µl	
-G/NTPs (25 mM each)	10.0 µl	
G (25 mM) 100 mM?	0.5 µl + 1.5 µl + water	(mu) (cat) (Rsr I)
Linearized cap (39 mM)	2 µl	1 1 1
DNA	(4 µg) 2-3-5 µl	
water	4 µl	
Σ volume	40 µl	1 1 1
		11 10 8

Uncapped

5 x	8		
25 mM NTPs	12	mu (2)	lassa (3) Rsr (5)
DNA	4		
Enzyme	13	12	10
water		4	1
H3 - ATP	40 µl		

RNA gel: ① lassa capped (as in 8/11/1994)
② RsrI capped
③ PI-5 capped
④ pM3000 capped

Ambion cap (39mM)

DNA

water

volume

5 X

25mM NTPs

DNA

Enzyme

water

H3 - ATP

2 µl 1 1 (RSV)

(4 µg) 2-3-5 µl

40 µl 1 1 1

11 10 8

A 014

uncapped

8

12 neg (2) (3) lassa RSV (5)

4

13 12 10

1 1 1

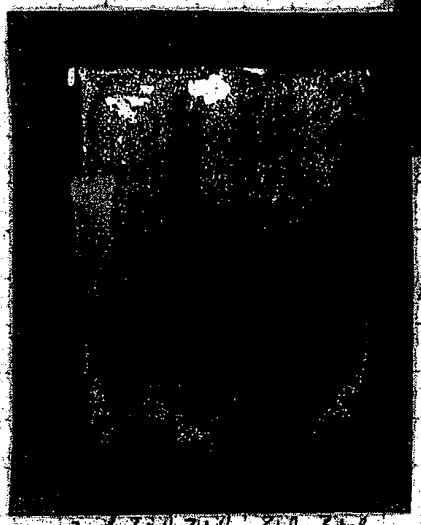
40 µl

RNA gel: ① lassa capped (as in 8/7/1994

② RSV1 capped — / —

③ P1-5 capped — / —

④ pV3000 capped — / —



3µl 3µl — 3µl —

LSV RSV V3000

Lassa Helper capped

capped capped

2µl of Promega's Ribomax

3µl 3µl 3µl 3µl 3µl

P1-5 LSV RSV P1-5 V3000

capped cap cap cap cap

from 8/7/1994

Transfection of BUK cells

Promega's RNA's

1.	pV3000	uncapped	(as PC)	10 µl (concen. see below)
2.	LSN	capped	(as NC)	10 µl
3.	LSN	— " —		{10 µl}
	Rsr	— " —		{10 µl}
4.	LSN	— " —		{10 µl}
	PI-5	— " —		{20 µl}
5.	PI-5	— " —	(as NC)	{20 µl}
6.	Rsr	— " —	(as NC)	{10 µl}

Promega's

LSN	100 ng/µl	cap +	10 µl	mistake occurred
Rsr	200 ng/µl	cap +	10 µl	
PI-5	100 ng/µl	cap +	20 µl	
L3000	200 ng/µl	uncap	10 µl	

Ambion's

LSN	200 ng/µl	cap +	10 µl
Rsr	200 ng/µl	cap +	20 µl
PI-5	200 ng/µl	cap +	20 µl

7.

	LSN	capped	{10 µl}
	Rsr	— " —	{20 µl}
8.	CSN	capped	{10 µl}
	PI-5	— " —	{20 µl}

5/9/94
Tuesday

Transcriptions (Promega's RiboMAx)

ATP, CTP, GTP, UTP	25mM each	5 (~3mM each)
T7	100mM	0.3 (~0.75mM)
Cap	39mM	2 (~2mM)
RNA Polymerase		2
		4
total vol.		40.3 µl

⇒ small amount of RNA
but probably a lot of capped!

Immunofluorescence

cells grown in the special vial of
grow medium removed
cells washed twice in ice-cold PBS 2 × 5 min
cells dryied
cells soaked in acetone for 10 min
dried
incubated with antibody: load ab as a thick layer
Lassa: ~120 µl undiluted
VEE: dilution 1:100 in 20% FBS (PBS)
Wash in PBS 20 min stirring and change in PBS
dry plates, lassa are ready for microscope.
VEE: second a-mouse fluorescein labeled anti-mouse IgG (H+L) (Goat) human.

G	100 mM	2 (~ 2 mM)
Cap	39 mM	2
RNA polymerase		4

Total vol. 40.3 µl

⇒ small amount of RNA but probably a lot of capped

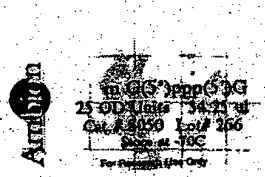
Immunofluorescence

cells grown in the special vial of
grow medium removed
cells washed twice in ice-cold PBS 2 × 5 min
cells dryied
cells soaked in acetone for 10 min
dried
incubated with antibody: load ab as a thin layer
Lassa: ~ 120 µl undiluted
VEE: dilution 1:100 in 20% FBS (PBS)
wash in PBS 20 min stirring and change in PBS
dry plates, lassa are ready for microscope
VEE: second a-mouse fluorescein labeled anti-mou IgG (H+L) (Goat) human serum adsorb
diluted 1:75

Photocopy Wednesday

Transcriptions (RiboMAX)

water	14
5X	8
MTP – G 25mM each	10
G 100 mM	0.5
cap 39 mM	2
DNA	2 (or 1 µl for pT-5 and pV3000 (+1 µl water))
polymerase	4
	40.5 µl



Amount of replicon VLPs

1. Trypsinize roller bottle with 5 ml of Trypsin-EDTA ~5' at RT C (~20°C)
2. Remove Trypsin-EDTA
3. Add 10 ml of Trypsin EDTA incubate ~6' at + RT (20°C) until cell just begin to come out of the surface (+5 ml of fresh Trypsin ~3 mn at R
4. Discard Trypsin EDTA
5. Add 10 ml EMEM, suspend to homogeneity by aspiration (~15 ml)
6. 2 ml into cell flask + 40 ml of EMEM
7. count the cells
8. 100,000 cells /per one cell (0.4 ml)

	dilutions 1	2	3	4	5	6	7	8
1. V 3000	1:100	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
2. Lassa replicon	(1:100)	(10^{-3})	(-4)					
3. CSN+RSR	10^{-2}	10^{-3}	etc					

G	100 mM	0.5
cap	39 mM	2
DNA		2 (or 1 µl for P1-5 and pV3000 (1 µl/µl water))
polymerase		4
		40.5 µl

Amount of replicon VLPs

1. Trypsinize roller bottle with 50 ml of Trypsin-EDTA ~5' at RT (~20°C)
2. Remove Trypsin-EDTA
3. Add 10 ml of Trypsin EDTA incubate ~6' at + RT (20°C) until cell just begin to come out of the surface (+5 ml of fresh Trypsin ~3 min at RT)
4. discard Trypsin EDTA
5. Add 10 ml EMEM, suspend to homogeneity by aspiration (~15 ml)
6. 2 ml into cape flask + 40 ml of EMEM
7. count the cells
8. 100,000 cells /per one cell (0.4 ml)

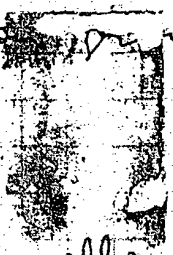
dilutions

	1	2	3	4	5	6	7	8
1. V 3000	1:100	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
5. Lasso replicon	(1:100)	(10^{-3})	(-4)					
2. RSN+RSP	10^{-2}	10^{-3}	etc					
3. SN+P1-5/P	10^{-2}							

(3) SN/RSP, (4) SN/P1-5/P

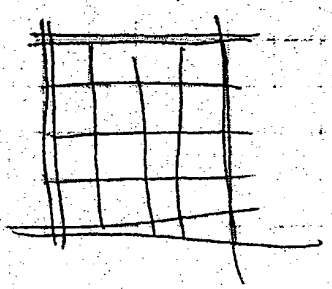
Tue 7th & morphology of cells

① V3000 — slowly grow, rounded cells
② LSN — quick grow, filaments
③ LSN + RSR — quick grow, — "
④ LSN + P1-5 — quick grow, — "
⑤
⑥
⑦
⑧ LSN + P1-5 — slow grow, rounded cells, as in ①



Calculation of cells

- 20 cells per smallest square
↓
$2 \cdot 10^5$ cells/ml
I need to seed ~ 100,000 cells per chamber
↓
400 µl into each chamber



Cell 8/11/94

Plate	Dilutions/chambers 1	2	3	4	5	6	7	8
1. V3000	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
2. LSN/RSR	"	"	"	"	"	"	"	"
3. LSN/P1-5	"	"	"	"	"	"	"	"
5. LSN	"	"	"	—— no ——				
4.	V3000 undiluted	LSN un-	LSN RSR un-	LSN P1-5 un-				
	10^{-3}	10^{-3}	10^{-3}	10^{-3}				

4/12/1996
Friday

IFA

1. Plates (Lab-Tek)
   Chambers and rubbers are removed,
   plates washed 3×5' in PBS
   (fairly cold)

2. Plates are dried on the bench
3. Plates are fixed in cold acetone
4. Plates are dried
5. Incubation with neu Cassa # 288 A6S conjugate (plates 1, 2, 3, 5) or mouse a-VEE A6 (plate 4)
   30 min
6. Wash in ice cold PBS 2×10 min on magnetic stirrer
7. Dry plates, Cassa are ready for microscope
   a-VEE (plate 4)
   100 µl of a-mouse conjugate
   1:?? in 20% FBS in PBS
   ≈ 0.5(?)
8. Plate 4 washed 20 min w/PBS
   dried
   + mounting media → microscope

a-Cassa: ① pV3000 all negative
② CSN/RSL
3 cells positive in 10^{-2} dilution
③ CSN/P1-5
2 cells positive in 10^{-2} dil
⑤ CSN all negative

a VEE ④ pV3000 – negative
LSN/RSL – negative
CSN – negative
LSN/P1-5 – positive

All cells are splitted.

15/1994
Monday

Description, (protocol from 8/10/1994)

Water	14
5 x	8
NTP-G	10
G	0.5
Cap	2 (pV300: 1 µl of Aurion + 2 µl Pharmacia 20 mM)
DNA	2 (1 µg P and V)
pol	4

BMK cells: 1) split and 2) electroporated (90-100% confluent, have clumps 70% - John F.)

Questions: 1) are clumped cells become normal monolayer after splitting?
.. cells be used

EMBL/MEDS PUSHKO
[illegible]

+10% Fetal Bovine Serum (20 µl) (20 µl) (20 µl)

Not enough cells for electroporation
200 ml - flask has about 1 x 10⁶ cells
I need 1 x 10⁷ per ml
and 500 µl per electropo

17/16/1994
Tuesday

BMK cell are 40-50% confluent

9/17/1994
Wednesday

Cells are ~80-90% confluent / 2 x 540 ml flask(s)

Electroporation BHK 21 with RNAs

Samples:

V.	(1)	pV300	for electroporation 2 x 540 µl flasks is just enough
R.	(2)	CSN/RSL	
P.	(3)	CSN/P1-5	
—	(4)	— (cells)	
cells	(5)	cells w/o electroporation	

(~1 µg) ~4 µg of RNA were used for each electroporation
Time const = 0.8 (!)

Cells were counted ~ 6×10^4/ml (Σ 40 ml) ⇒ $2.4 \cdot 10^7$ cells
Resuspended in 2 ml of ice-cold PBS
~ 1.0×10^7 cells/ml (as in protocol)
Electroporation, as described in protocol
Electroporated cells were seeded into either
LABTEK covers slips or small flasks at 10:30 am
(L) or (V) antibody

	V	R	P	—
	V	R	P	cells

400 µl/chamber or 100 µl/chamber for tomorrow morning (+30 µg/ml medium)
or 900 µl/chamber for analysis at 4:30 pm (6 hr post synch)

Remaining cultures into flasks and grow 24 hrs (10:30 ÷

BHK cells (~70% confluent 1 x 540 ml/flask)
trypsinized
cells counted = $5 \cdot 10^4$/ml

~20-25 thsd. of cells per chamber of LABTEK slips

1 L of 10x PBS
- 80 g NaCl
- 2 g KCl
- 11.5 g $Na_2HPO_4 \cdot 7H_2O$ (14.4 g Na_2HPO_4)
- 2 g KH_2PO_4 (12.5 g $K_2H_2PO_4$?) 0.95 g K_2HPO_4

2 l

Slides of Vs and Ls were taken after 6 hrs incubation and IFA was done as described in protocol, with anti-Lassa and anti-VEE antibodies

Simultaneously, after 6 hrs p.e. (post electroporation) 500 µl of supernatants were taken and frozen at -70°C (From flasks)

Result: 1) less than 1% of cells were positive for Lassa antigen after 6 hrs p.e. 5-10 cell per vie
2) roughly 1% are positive for VEE antigen 10-20 cel per vie

8/18/94 Thursda

IFA of cells electroporated 8/17/1994

a-Lassa: 30 hrs p.e. ~1 (max 10)% of cells expres Lassa antigen not pV3000 or NC.

a-VEE: 30 hrs p.e. ~1 (max 10)% of cells expres VEE antigen / Rst & pV-5 helpers; pV 3000 (ecrit) / no VEE antigen (-) controls / electroporated w/o RNA cells

Electroporation efficacy is low (1÷10%

Supernatants were taken from transfected cells 6 and 30 hrs p.e, diluted 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}:

10^{-1}	10^{-3}	10^{-5}	10^{-7}
-1	-3	-5	-7
10	10	10	10

100 µl of sup +	900 µl EMEM	②
10	990	②
10	990	③
10		

NC	-1	-3 / -5
	-7	

400 µl of such mixtures were put to chambers of 8-chamber Lab Tek plates (Nunc) incubated 18 hr (0/N) 8/19/94 Friday

IFA for lassa antigen of cells infected with replicon containing VLPs (RVLPs)

$V_{6\,p.e.}$ = "0 cells"; $V_{30\,p.e.}$ = " " at all dilutions (10^{-1}, 10^{-3}, 10^{-5}, 10^{-7})

2) for lassa antigen after 6 hrs p.c. per viable cell roughly 1/6 are positive for VEE antigen — 10-20 cells per viable

A 025

8/18/94 Thursday

IFA of cells electroporated 8/17/1994

a- Lassa : 30 hrs p.c. ~1 (max 10)% of cells express lassa antigen, not pV3000 or NC.

a- VEE : 30 hrs p.c. ~1 (max 10)% of cells express VEE antigen (Rst + PI-5 helpers); pV3000 (ccIII); no VEE antigen in (-) controls / electroporated w/o RNA cells

Electroporation efficacy is Low (1÷10%)

Supernatants were taken from transfected cells 6 and 30 hrs p.c, diluted 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}:

P_{30} V_{30} R_6 P_6 V_6

	10^{-1}	10^{-3}	10^{-5}	10^{-7}
	-1	-3	-5	-7
	10	10	10	10

NC	-1	-3	-5	-7

100 µl of sup + 900 µl EMEM

10 990 ↓
10 990 ↓
10 990 ↓

400 µl of such mixtures were put to chambers of 8-chamber (at Tek plates (Nunc) incubated 18 hr (O/N)

8/19/94 Friday

IFA for lassa antigen of cells infected with replicon containing VLPs (RVLR)

$V_{6\,p.e.}$ = „0 cells"; $V_{30\,p.c.}$ = „0" at all dilutions (10^{-1}, 10^{-3}, 10^{-5}, 1
$R_{6\,p.e.}$ = „0"; $R_{30\,p.e.}$ = „3" at 10^{-1} dilution; „0" at other di
$P_{6\,p.e.}$ = „0"; $P_{30\,p.e.}$ = „10" at 10^{-1} dilution, „0" at other di
„ $_{30\,p.e.}$ = „0"; „ $_{6\,p.c.}$ = nt at all dilutions

20

To increase efficiency of transformation

Transcription of pH3000 (5X) 26/16 water

NTR-6 22

Cap 4 of Pharmacia (20mM)

DNA 1.5

pel 8

G 2

80µl

To (40)µl/transfection

per 400µl culture

(5X10^5) bacteria (5X10^6)

8 X 10^5 bacteria

pheletopen 6,500µl ⇒

6 X 10^5/ml

Time constant 0.7 for 2.5 kV - culture ~ 400µl

0.9 for 1.5 kV - culture ~ 350µl

9.5 ml EMEM + 0.45 ml electroporated cells

↓ 400µl ↘ small flask (30ml)

2 of 8-chamber Lab Tek

GIBCO BRL

Store at -5°C to -20°C

2-28-05

Try EDTA

1X

(0.05% ... 0.53 mM EDTA-4Na)

porcine ... tested

mycoplasma tested

Prepared with 0.5 g Trypsin (1:250) and 0.2 g EDTA·4Na/L in ... Ca++ and Mg++.

Product contains water (CAS No. 7732-18-5).

Cat. No. 25300-013 Lot No. 15P4340

For research use only. ... 610-5300mL

For Cell Culture or Further Manufacturing Use

LIFE TECHNOLOGIES

21

IFA of cells electroporated 8/19 — 8/20/1994 Saturday

Cells were electroporated w/ pV3000 RNA ⇒ all must be positive since virus produced by transfected cell is infectious and should infect untransfected cells.

IFA according to protocol, with alteration of protocol: after first Ab slides washed 2 times w/PBS (according to protocol, should be 3 times) after conjugate slides washed 3 times w/PBS (according to protocol, should be 4 times)

In both cases (e.p. with either 2.5 kV or 1.5 kV) VEE-positive cells constitute ~ 5% of total cell population (no difference in transfection efficacy for 2.5 kV and 1.5 kV)

May be, they became "equal" (same percentage of VEE-positive cells) after transfection, due to infection with produced virus?

8/21/94 Sunday

Transcription Ambion mMessage m Machine

(50)(55) H2O (GSN)(P1-5)
20 µl 10× Buffer
100 2×
10(5) DNA GSN(P1-5)
20 10× Enz
200

Cells infected w/ pV3000 RNA and supposed to produce infectious virus seem to grow well, without visible alterations? May be, cells e.p. at 2.5 kV are not filamentous, rather round-shaped, with "pili"

BHK cells are splitted for tomorrow

5/22/1994 Monday

Electroporation

BHK cells are grown to ~ 80-90%
Cells 2.7 · 10⁷ cells/ml
resuspended in 2.5 ml ⇒ 1 · 10⁷ cells/ml

Samples:

1. 1.5 kV; as in protocol
2. 1.5 kV; 37°C after e.p. (10')
3. 1.5 kV; + EMEM (37°C), 10' at RT
4. 1.5 kV; 5 times; 10' RT
5. 2.5 kV; 2 times; 10' RT

(1–5): 20 µl CSN RNA + 20 µl HeLa + 450 µl cells

6. cells, no RNA, 2.5 kV 5 times; 10' RT

10 40 incubation at 37°C started

(6 hrs)

16 00

21 40 (11 hrs)

a-lassa

мало клеток не прикрепл.

1. 1:100 more/IFA positive ~30% of live cells are positive
2. ~100% ~40-50% are positive
3. ~100% ~5% are positive
4. ~10% a bit of dead
5. ~10% — dead —
6. ~1% all cells are dead

мало прикрепл. клеток

от. много убитых клеток

мало клеток прикрепилось, но много мертвых

много прикрепл. клеток

if 1 = 100% of cells (suppose)

36/200 18%
31/140 22%
positive/total cells

the cells attached to the bottom (4)(5)(6) show only ~10% in comparison to (1)(2)(3)

IFA

to be filtered

to be

rather round-shaped, with pro

A 029

BHK cells are splitted for tomorrow

5/22/1995
Monday

Electroporation

7150 BHK cells are grown to ~ 80-90%
Beads 2.7 · 10⁷ cells
resuspended in 2.5 ml ⇒ 1 · 10⁷ cells/ml

Samples:

1. 1.5 kV ; as in protocol
2. 1.5 kV; 37°C after e.p. (10')
3. 1.5 kV; + EMEM (37°C), 10' at RT
4. 1.5 kV; 5 times ; 10' RT
5. 2.5 kV; 2 times ; 10' RT

(2)–(5): 20 µl CSN RNA + 20 µl H₂O + 450 µl cells

6. cells, no RNA, 2.5 kV 5 times; 10' RT

10 40 incubation at 37°C started (6 hrs)

16 40

a-lassa

мало клеток не прикрепил.

1. not all cells
2. ~100% ~ 40-50% are positive
3. ~100% ~ 5% are positive
4. ~10% a lot of dead
5. ~10% — dead —
6. ~1% all cells are dead

мало прикрепившихся клеток

мало живых клеток

if 1 = 100% of cells (suppose)

36/200 18%
31/140 22%
positive/total cells

If the cells attached to the bottom (4)(5)(6) more only
~ 10% in comparison to (1)(2)(3)

many unattached cells in all samples

21 40 (11 hrs)

after cel

мало клеток прикрепилось, но много живых

yellow-green

морфол. – округл.

большинство мертвых, мало клеток прикрепилось

практически все мертвые

Cells откл

on slide

IFA

to be

Protocol for electroporation of BHK-21 cells:

Reference: Liljeström et al., J. Virol. 65:4107-4113 (1991).

Cells: BHK-21, low passage number from ATCC. Culture medium: MEM+NEAA+10% FBS or DMEM + 7.5% FBS. When passaging or preparing cells for electroporation, **BHK monolayers should never be allowed reach complete confluence.** This causes clumping and is generally irreversible. If this happens, throw them away and start over. Note that you will need 5 x 10^6 cells per electroporation, use cells that are 60-80% confluent.

Apparatus: BioRad Gene Pulsar™ with Pulse Controller. 0.2 mm electrode gap cuvettes from BioRad. Other brands of electroporation devices probably will also work but may require adjustment of settings (voltage, capacitance, etc.) to optimize transfection efficiency and cell survival.

RNase-free PBS: Cells are washed and shocked in this solution. It is advisable to check the electroporation time constant of your PBS before starting, the value should be 0.7 at the settings given below.

1. Wash the cell monolayers twice with PBS.

2. Add 3 ml trypsin per 150 mm plate. Incubate at 37°C for ~ 5 min until trypsinization is complete. Gently produce a single cell suspension in the trypsin solution using a pipette and transfer the cells to MEM + 2% FBS to stop trypsinization (10 ml per plate).

3. Collect the cells by low speed centrifugation at room temperature. 500 rpm, 5'

4. Carefully remove the supernatant and resuspend the cells in ice-cold RNase-free PBS (we use 3-5 ml per 150 mm plate). 10 ml

5. Determine the yield of the cells (we usually count the number of cells diluted 1:10 in PBS).

6. Collect the cells as in step 3. Wash one additional time with ice-cold RNase-free PBS (repeat steps 4 and 3).

7. Resuspend the cells at a concentration of 1 x 10^7 cells/ml in ice cold RNase-free PBS and keep them on ice.

8. Put the RNA samples to be electroporated in a 1.5 ml microcentifuge tube on ice. Add 500 µl of the cell suspension, mix gently, and quickly transfer the RNA/cell mixture to the electroporation cuvette. Put the cuvette in the holder of the electroporation device.

9. Shock the cells two times using the following settings. 1.5 kV, 25 µFD, with the pulse controller resistance set on infinity. The time constant should be approximately 0.7. Cell survival is typically 65-75%.

10. After the electric shocks, allow the cells to sit at room temperature for 10 min. After this recovery period, the cells are diluted 20-fold with MEM + FBS (9.5 ml) and plated. We usually use 0.5-2 ml/ 24 mm well, 1-4 ml/ 35 mm plate, 4-6 ml/ 60 mm plate, or 8-10 ml/ 100 mm plate, depending upon nature of the experiment.

.2 cm

Mette - most recent procedure from Bob's lab looks like it's the same as before - but probably should check

4 hrs p.e. 10 µl of supernatant was taken and frozen a
The slides were subjected to IFA w/ a-VEE.
~1:2⁰/₀ positive

6 hrs p.e. a-VEE and a-Lassa
~30⁰/₀ positive ~20⁰/₀ (+)

8 hrs p.e. a-VEE (2x)
not clear, there are positive cells: either all positive, only few

10 hrs p.e. washed, dryed, aceton treated (10'), dryed,
a-VEE a-Lassa
not clear ~20⁰/₀ positive

8/26/1994 Friday 24 hrs p.e. a-VEE
not clear, too many cells,
which probably make antigens
within the cells inaccessible to antibodie

BHK cells are growing for
electroporation (~40⁰/₀ confluent)

8/28/1994 Sunday Electroporation

{ ① 25 µl CSN + 25 µl PI-S as in protocol
② 1÷3·10⁷ cells/ml before electroporation 1.75 kV and 10 a
10⁴⁰ ÷

BHK seeded on Lab-Tek slides at
Buechler cone. 1.2·10⁵ cells per chamber of 8-c
(Lab-Tek

6 hrs p/e a-VEE ① ~25⁰/₀ positive
② ~20⁰/₀ positive

8 hrs p.e. a-VEE (2x)
not clear, there are positive cells: either a positive only [illegible]

10 hrs p.e. washed, dryed, aceton treated (10'), dryed, [illegible]
a-VEE not clear
a-lassa ~ 20% positive

8/26/1994 Friday 24 hrs p.e. a-VEE
not clear, too many cells, which probably make antigens within the cells inaccessible to antibodi

BHK cells are growing for electroporation (~ 40% confluent)

8/28/1994 Sunday Electroporation

{ ① 25 µl CSN + 25 µl PI-5 as in protocol
② 1 ÷ 3 · 10⁷ cells/ml before electroporation 1.75 kV and 10 µF
10⁴⁰ ÷

BHK seeded on Lab-Tek slides at Quecken conc. 1.2 10⁵ cells per chamber [illegible]

6 hrs p/e a-VEE
① ~ 25% positive
② ~ 2% positive

a-lassa
① ~ 15% of cells are positive
② (1.75 kV; 10 at 37°C) ~ 10% of cells are positive

supernatants are frozen (10µl and 1.5 ml aliquots)

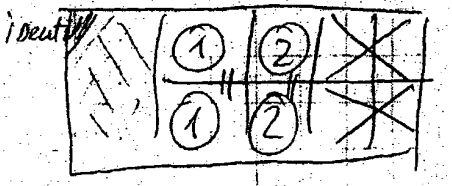
48 hrs p.e. Slides are washed w/PBS 10' and dryied, kept o/n at -20°C.

72 hrs p.e

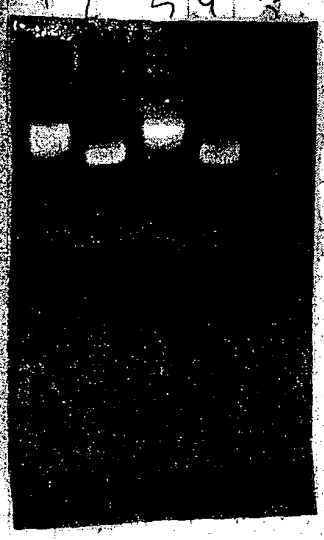
8/29/94 Monday

96 hrs p.e. w/o o/n freezing (of course)

IFA w/ a-VEE and a-Lassa



Plasmid DNA cleavage w/ NotI



① LSN
② BSR
③ pV3000
④ pI-5
⑤ K6 ladder

} ~1µg/well (in 40µl)

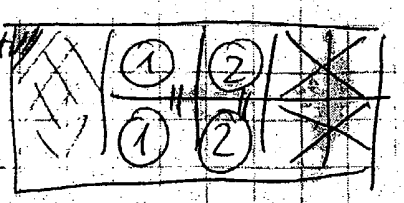
a-Lassa

p.e., hrs	number of positive cells (mean)	Total no. of cells (mean)	positive cells (percent)
6	1) 60; 40; (50)	ND	ND
	2) 39; 20; (29.5)	ND	ND
9	1) 85; 83; 60; 68 (74)	ND	ND
	2) 67; 50; 43; 60 (55)	500	12%
12	1) 76; 88; 80 (81)	ND	ND
	2) 90; 46 (68)	500; 400	18% 11.5%

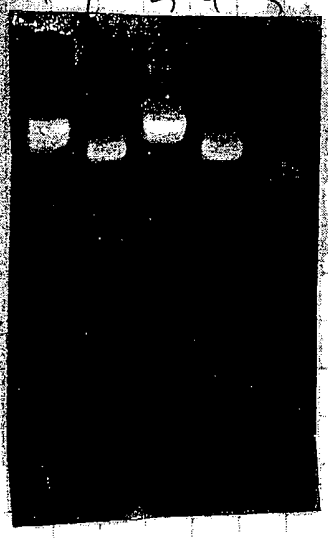
8/24

22 hrs p.c. w/o f/t freezing (of course)

IFA w/ a-VEE and a-Lassa



Plasmid DNA cleavage w/ NotI



① LSN
② BSR
③ pV3000
④ p1-5
⑤ K6 ladder

} ~1.7μl/well (w/ 40μl)

a-Lassa

p.c. hrs	number of positive cells (mean)	total # of cells (mean)	positive cells (percent)
6	1) 60; 40; (50)	ND	ND
	2) 34; 20; (29.5)	N.D	ND
9	1) 85; 83; 60; 68 (74)	ND	ND
	2) 67; 50; 43; 60 (55)	500	12%
12	1) 76, 88; 80 (81)	ND	ND
	2) 90; 46 (68)	500; 400	18%; 11.5%
22	1) 70; 65; 52 (62.3)	600	8.7%
	2) 100; 58 (79)	600; 200	16.6%; 29%

T 2(

a-VEE

p.e./hrs	no. of positive cells	total no. of cells	positive cells (percent)
6	1) 34; 45 (42)	ND	ND
	2) 36; 38 (37)	ND	ND
9	1) 35; 7 (21)	ND	ND
	2) 19; occasional	200	9.5%
12	1) ~18; 14 (16)	250	5.6%
	2) 18; occasional	~~200~~ ND	ND
22	1) 23; numerous	ND	ND
	2) 10; numerous	~~100~~; many	2%

40 x / 0.75 objective
10x / 25 diaphragm

8/29/94
Tuesday

RNAs : transcription rxn

LSN
RSV
VEE
P1-5

		uncapped	capped	capped
Promega kit	H2O	~~12~~	water	~~12~~ 23
5SN	5x	8	5X	16
transcription	NTP	12	NTP-G 10	20
	DNA	~~4~~ (?)	G 0.5	1
	pol	4	Cap 2	8 (20mM)
		40µl	DNA	~~4~~ 4
			pol ~~4~~	8
			40 µl	80µl

m7G(5')ppp(5')G
Z7-4635-02 25 u
Lot 31[illegible] 5021
Store at C
Made in USA
Promega

6hrs post infection 1.5kV; 2times; 10'RT

	0	3	51	104
	6hrs p.e.	9hrs p.e.	12hrs p.e.	24hrs p.e.
10^{-2} dilution	ND	0	43	123

1.75kV; 2times; 10'37°C

10^7 cells/ml electroporated cells

Amount of positive cells/well = particles/ml

1.5kV; 2x; 10'RT

ND	ND	34	88
4hrs p.e.	6hrs p.e.	10hrs p.e.	24hrs p.e.
ND	ND	66	127

10^{-2} dilution

1.5kV; 2x; 10'37°C

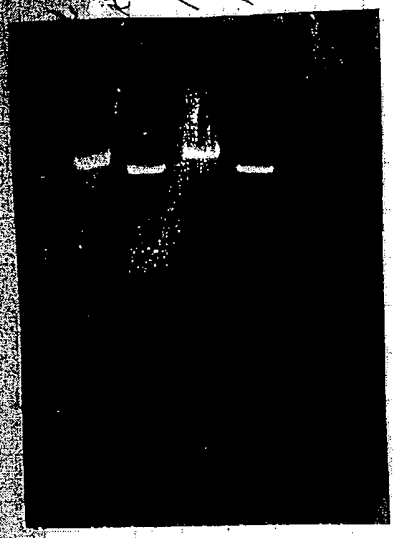
Infection with replicon pa

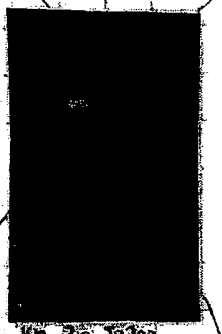
Media from BHK cells removed by vacuum, add 100 µl of 10^{-2} d of transfected cells,

Media added (300 µl) continued at 37°C

Cleaved DNA

NotI





A 036

see next page

from capped RNA (80µl) ~~50~~ µl has been taken,
+ 20 µl LiCl Precip-Sol'n
at least 30min - 20°C (2hrs in reality)
is min at max rpm,
wash 75% EtOH

dry, dissolve in 30µl water

28

left to right:
① LSN uncapped w/DNA (2x)
② -"- capped w/DNA (1x)
③ -"- -"- w/o DNA
④ PI-5 capped/ambion

} 1 µl of each

LiCl removed

8/31/1994 IFA of infected SUK cells: a-Lassa infected with replicon LSN + PI-5 helper, 24hrs p.i. (post-infect

N	ND	ND	118	488	1.5kV; 2x; 10' RT
dilution 10^{-2}	~~6 hrs p.e.~~	~~9 hrs~~	~~12 hrs~~	~~22 hrs~~	
	ND	ND	134	529	1.75kV; 2x; 10 37°

24hrs post infection

10^9 cells/ml for electroporation

24 hrs post infection

see 6 hrs post infection on a previous page

10^9 cell/ml for el.porat.

0	~~4 hrs p.e.~~	0 hrs p.e.	193	475	1.5kV; 2x; 10' RT
dilution 10^{-2}	~~11~~	0	228	~~479~~	1.5kV; 2x; 10' 37°

Electroporation

~~(1) 10 µl of LSN uncapped (Fig. p. 28, lane 1) + 5 µl H2O,~~
~~(2) 10 µl of capped RNA with DNA and nucleotides (lane 2)~~
~~(3) 10 µl of capped purified RNA (lane 3)~~

① 15 µl of lane ① + ~~H2O~~
② 5 µl of lane ② + 20 H2O
③ 35 µl of lane ②
④ 3~~5~~ µl of lane ③ + 20 H2O
⑤ 25 µl of lane ③
⑥ 15 µl of lane ① at 37° 10'

12% efficiency (5-10%)
25-30% (40%)
18-21% (50-60%)
~~50-65%~~
~~50-85%~~

Time 0.3 !?
Time const 0.3 ?! (~10%)
Набирает, нарастает медленно
0.8 = 0.3 ?!

incubation at 37°: 8 10 ÷ 7 30 (~13 hrs)
started

4/11/94
Thursd

IFA with α-Lisa conjugate

Transkriptions

	① LSN - Promega	② P1-5-Promega	③	③ LSN - Ambion	④ P1-5-Ambion
Water	23	23	23	Σ 80 µl	Σ 80 µl
5x	16	16			
0.25mM NTP-G each	20	20	20		
G 100mM	0.5	0.5			
Cap 20mM	8	8			
DNA	4 (LSN)	4 (P1-5)			
µl	8	8			
	Σ 80 µl	Σ 80 µl			

(as described in manual)

40 µl + 20 µl LiCl 30' at -20°C, ct, wash, dry, dissolve in 3

~~15~~ mM each C, U, A
n.p.m

~~10 µl of capped RNA with DNA and nucleotides (lane 2)~~
~~10 µl of capped purified RNA (lane 3)~~

① 15 µl of lane ① + ~~10 µl~~
② 5 µl of lane ② + 20 µl
③ 35 µl of lane ②
④ ~~35~~ µl of lane ③ + 20 µl
⑤ 25 µl of lane ③
⑥ 15 µl of lane ① at 37° 10'

Time 0.3 ?!
Time const 0.3 ?! (≈ 10')
Inkubation, Inkubator, Inkubation
0.8 = 0.3 ?!

12% efficiency (5-10)
25-30% (~~40~~ 50-6
18-21%
~~50-55%~~
50-55%

incubation at 39 : 8 10 ÷ 7 30 (~ 13 hrs)
started

9/11
Thurs

IFA with a-lasa conjugate

Transkriptions

	① CSN - Promega	② P1-5 - Promega	③	③ CSN - Amersham	④ P1-5 - Amersham
water	23	23		Σ 80 µl	Σ 80 µl
5x	16	16		(as described in Name	
0.25mM NTP-G	20	20			
2mM	0.5	0.5			
Cap 20mM	8	8			
DNA	4 (CSN)	4 (P1-5)			
µl	8	8			
	Σ 80 µl	Σ 80 µl			

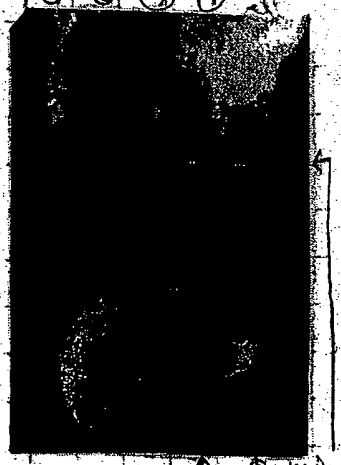
40 µl + 20 µl Cill 30' at -20°C, CT, wash, dry, dissolve

~~15~~ mM each C, U, A
0.6 mM G
2 mM Cap

30

9/2/1996
Friday

RNA gel



1 Amb LSN Lic1 ⊕
2 Amb P1-5 Lic1 ⊕
3 Prom LSN Lic1 ⊕
4 Prom P1-5 Lic1 ⊕
5 Prom P1-5 Lic1 ⊖

← minor (~20 ng) band of RNA
⊕ degradation of RNA

IFA with 1) α-Lassa } 12 hrs p.e
2) α-VEE } 18 hrs p.e

unpurified

1 Amb LSN Lic1⁻
2 Amb P1-5 Lic1⁻
3 Prom LSN Lic1⁻
4 Prom P1-5 Lic1⁻
5 Amb LSN Lic1+

20 ng/µl
50 ng/µl
100 ng/µl
150 ng/µl

purified

1 Amb LSN Lic1+
2 Amb P1A5 Lic1+
3 Prom LSN Lic1+
4 Prom P1-5 Lic1+
5 Amb LSN Lic1⁻

20 ng/µl
50 ng/µl
100 ng/µl
150 ng/µl

400 µg
1 µg
2 µg
3 µg total

Interesting: relative amount of Sassa-positive cells is much higher in 18 hrs p.e than in 12 hrs p.e. Whereas number of VEE-positive seems to be the same

Does this measurement mean transfection with replicon particles?

TRANSFECTION

Electroporation 9/1/1994

Samples

1. ① A UCl : Ambion 10µl LSN + 10µl PI-5 ; DNA⁻, cap⁺
2. ② A : — / — / — / — ; DNA⁺, cap⁺
3. ③ P UCl : Promega 10µl LSN + 10µl PI-5 ; DNA⁻, cap⁺
4. ④ P : — / — / —
5. ~~5.~~
6. ~~6.~~
7. ~~7.~~
8. ~~8.~~

A 041

Conclusions:

1) Наверное, было мало RNA, (~ 1 µg/мл мешали)
2) После пассажа больше клеток (~ ×10⁷/ml), но вместе больше трансфицированных
3) µg - +: RNA degrad.
4) перегружали?!

1	24/180	13	2	32/200	16
	50/200	25		18/200	9
	55/200	28		43/200	22
		22%		48/200	16
					16
3	0/0	0	4	0/150	
	3/43	7		0/200	
	10/200	5 4%		3/200	2 1%

IFA 9/2/1994

12k 18 hrs p.e.

① and ③, especially ③

1: 6% / 22%	2: 5% / 16%
3: 5% / 4%	4: 2% / 1%

Antibody

Conjugate
a-Sassa #288

mean 12 hrs p.e % of positive cells / mean 18 hrs p.e % of positive cells

a - Sassa, 18 hrs p.e

a - VEE, 18 hrs p.e

1: 14% / ~same	2: 17% / ~same
3: 1% / ~same	4: 0.5% / same

Antibody
a - VEE

Conjugate
a - mouse

1 2 3 4 Capsid Same amounts as in 12 hrs p.e.

~~Transfection w/supernatants from ab~~

Antibody

Conjugate
a-Sassa #288

Inserted between pg 30 + 31

RNA **TRANSFECTION**

Electroporation 4/4/94, at room t°, 10' at 37°C p.c.

Samples
1. 30 µl PI-5 (Amblon), unpurified
2. 20 µl CSN (Amblon), purified
3. 10 µl CSN (Amblon), unpurified
4. 60 µl + ~450 µl of cells (~ $1 \cdot 10^7$/ml)
5. ~~
6. ~~
7. ~~
8. ~~

IFA 9/5/94 PBS washes: 5' before & after acetone; 10' after conjugate

Antibody ~~
Conjugate α-Lassa #288

14 hrs p.e.	33/200 17% 24/170 14% positive cells / total # of cells			
	31/300 10%			

mean of positive cells (14%)

IFA 9/5/94 PBS washes: 5' before & after acetone; 5' after Ab; 10' after conj.

Antibody mouse α-VEE
Conjugate α-mouse

14 hrs p.e	51/200 26% 22/200 11% 30/200 15%			

mean (17%) ⇒ only 850,000 cells (out of 5,000,000) may produce VLPs

Infection 9/5/94 (dilution 10^{-2}) ① 14 hrs p.c ② 16 hrs p.c.

IFA 9/6/94 hrs p.i.
Antibody ~~
Conjugate α-Lassa #288

1) 14 hrs p.e 2) 16 hrs p.c young cells	① 0/1	② 46/3		
1) 14 h p.c 2) 16 h p.c old cells	① 4/0	② 12/4		

(~10^2 particles/ml) (10^2–10^3 particles/ml)

9/4/94
Sunday

Transfection

Preparation

30μl of P1-5 Amara (8/21/94)
20μl VLSN/well + 10μl Ambion ×
at RT; 10' at 37°C

9/5/94
Monday
Saturday

IFA a-Lassa (~14% efficiency)
a-VEE 17% efficiency

VSN cells (young, 2 days; and old, 4 days)
infected w/ replicon particles

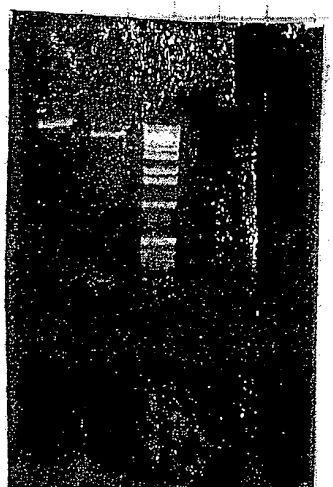
IFA a-Lassa
(of cells infected with replicon particles)

9/6/94
Tuesday

Transcriptions (on Message m Machine)

Water	20μl	~~Salts~~	17
10× buf	8	10	8
2× NTP/cap	40		40
~~P~~ VSN	4	P1-5	(7)
10× Enz	8	10 Enz	8
	80μl		80μl

37°C 1½ hr



Mutiny (1)μl resuspended

(2)μl

50µl of PI [illegible] 1%
20µl VLSN/well +10µl Ambion ↑

at RT; 10' at 37°C

2/15/94 Monday (Saturday)

IFA a-Lassa (~14% efficiency)
a-VEE 17% efficiency

VSN cells (young, 2 days; and old, 4 days) infected w/ replicon particles

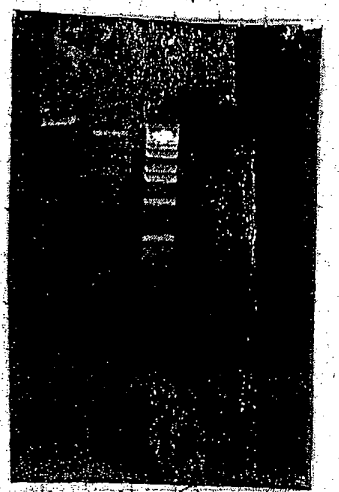
4/16/94 Tuesday

IFA a-Lassa
VSN cells, infected with replicon particles

Transcriptions (in Message m Machine)

water	20µl	~~water~~	17
10x buf	8	~~10~~	8
2x NTP/cap	40		40
~~P~~ VSN	4	PI-5	(7)
10x Enz	8	10 Enz	8
	80µl		80µl

37°C 1h



RNA gel ① VSN
② PI-5 } Ambion, non-purified

Urgent (1) µl comparison
(2) µl

32

7/7/94
Wednesday

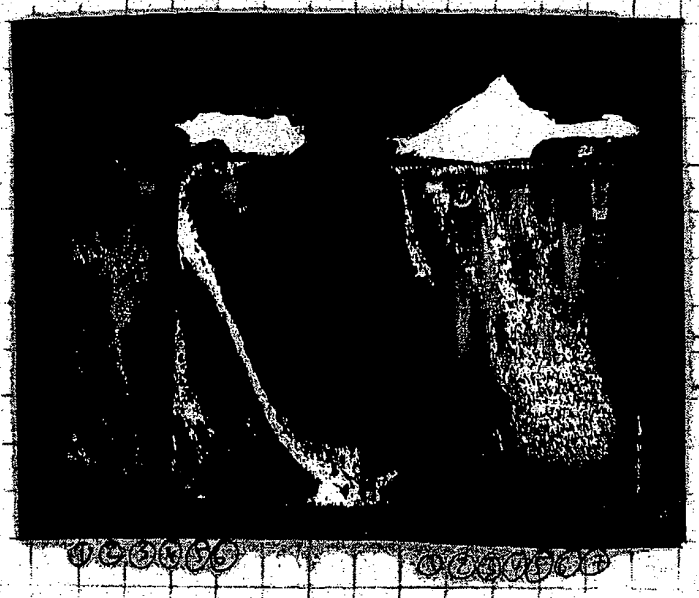
DNAse I (24/µl, Qiagen mMgMeOH)

RNA LsN	9/6/94	5 µl	0.25 µl	37°C 15'
RNA P1-5	9/6/94	5 µl	0.25 µl	37°C 15'
RNA P1-5	8/21/94	5 µl	0.25 µl	37°C 15'

(1.5 µl) + 0.5 µl + 0.5 µl + 2.5 µl + (1.5 µl EtBr) + (+ 1.5 µl BPB)
RNA 10x PB Formamide Formalin

(ottavio) → -20°C for DNA gel

RNA gel : DNA gel :



			RNA gel	DNA
1.	LSN 9/6/94	T7 trascription mixture	1.5 µl of reaction	3.5 µl
2.	P1-5 9/6/94	T7 trascription mixture	1.5 — / —	3.5 µl
3.	P1-5 8/21/94	T7 trascription mixture	1.5 — / —	3.5 µl
4.	LSN 9/6/94	T7 trascription + DNAse	1.5 — / —	3.5 µl
5	P1-5 9/6/94	T7 trascription + DNAse	1.5 — / —	3.5 µl
6.	P1-5 8/21/94	T7 trascription + DNAse	1.5 — / —	3.5 µl
7.	λ BstE II			

33

9/7/1994

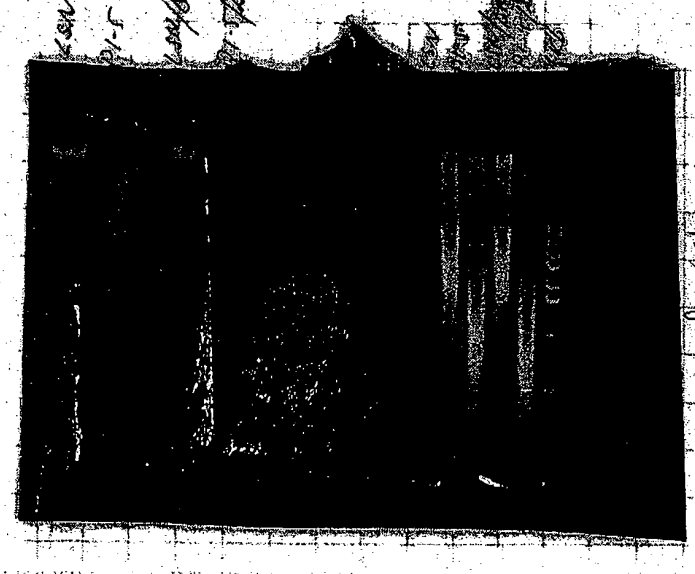
Transcriptions: mMmM (another)

	LSN		PI-5
Water	8		9
10x But	4		4
2x NTP/cap	20		20
GTP 30mM	2	(67% capped)	1.0
LSN	0.5	PI-5	1
10x Buf	4		4
3H-ATP	1		1
	40		40

PI-5 variant

Water	~~8~~ 7
10xbut	4
2x NTP/cap	20
GTP 80mM	1.5
PI-5 DNA/Not	2.5
10x Buf	4
3H-ATP	1
	40µl

6.26%



PI-5/Not I cuts pAg phage, precipitate

1 [illegible] QUENCH PRESET [illegible] WED 07 SEP 1994 15:24

FILE RE[illegible] CYCLE REPEAT [illegible] SCR:N [illegible]

ADC PROGRAMMING ROM:N

CHANNEL [illegible] UP: 400 2STGMA: 0.50 LRG SUB: 0.00 BKG 2STG: 0.00 LSR:

[illegible] UP ON: 29 NOV 1990 12:57

[illegible] SUBTRACTIONS:

[illegible] SUBTRACTION

[illegible] SUBTRACTION

[illegible] BACKGROUND QUENCH CURVES:N BACKGROUND QUENCH CURVES:N

[illegible] QUENCH LIMITS LOW:-2.67 HIGH:29[illegible]

[illegible] 000

34

Electroporation RNAs

19.00

① 10 µl of P1-5 8/21/94 + 10 µl of LSN 9/7/94

② — " — / — / — / — — + purified

(20 µl + 5 µl NH4Ac,
75 µl cold EtOH
15' -70°C
15' spin down,
wash w/ 75% EtOH
dry, dissolved in 20 µ

settings: as in protocol,
with exception: 1.75 kV
10' at 37°C p.e.

4/8/1994
Thursday

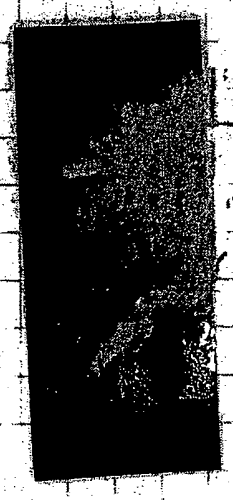
IFA: a-Lassa 45% positive
a-UEE 47% positive

8:00 - - supernatant

12:30 1 ml of ① ② } supernatants → -70°C

17:00 2 ml of ① supernatant → -70°C

Depletination of P1-5/NotI:



BHK cells are seeded (~ 50,000/well-chamber of 8-ch
12 ml from roller bottle (5 ml of Trypsin + 30 ml EMEM
per T150 flask ~ 1.5 · 10⁶/ml cells

TRANSFECTION

Electroporation: 9/7/94; at RT, 1.75 kV, 10' at 37°C p.e.

Samples: 1 10µl of P1-5 RNA 8/21/94 + 10µl of Lsn RNA 9/7/94
2 same RNAs, but precipitated before electroporation (to remove NTPs)

purification by precipitation w/ ethanol and NH_4Ac drastically decreased transfection efficiency

9/14/94

EA positive/total Antibody Conjugate a-Lassa 1:2211	1 38/94 (40) 20/37 (54) 10/25 (40) 11/23 (48) 22/47 (47)	2 2/40 (5%) 5/40 (13)	—	—
	1 9/24 (38) 17/41 (41) 17/30 (57)	2 1/20 (5) 1/23 (4)	—	—
	mean (45)%	(7)%		

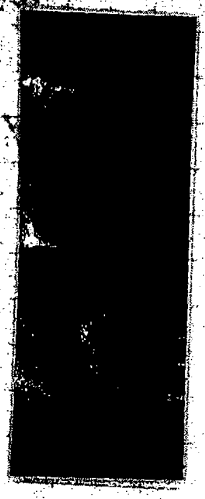
VE Antibody a-VEE Conjugate a-mouse 1:1hr	1 15/38 (39) 2 24/38 (63) 14/39 (36)	7/20 (5) 2/30 (7)	—	—
	1 20/29 (69) 8 11/27 (41) 18/50 (36)	3/40 (8) 2/20 (10)	—	—
	Mean (47)%	(8)%		

Infection

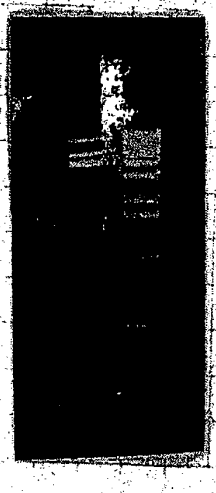
		18 hrs	36 hrs	60 hrs
Antibody Conjugate a-lassa	—	10^{-2} dilution 33	10^{-2} dilution 123	10^{-2} dilut 125
	—	10^{-4} not detected	10^{-4} 1	10^{-4} not detected

pV 3000 : EcoRI ApaI (Reast 4)

DNA	100 µl	(~10 µg)
10x Buf ④	50 µl	
Water	340 µl	
EcoRI	6 µl	(60 u)
ApaI	6 µl	



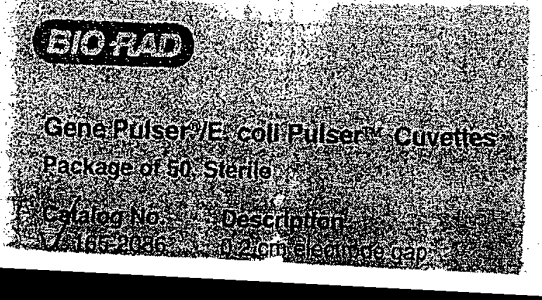
⑥ hrs, + 50 µl Reast ③
+ 450 µl water
+ 5 µl EcoRI
overnight



9/10/94 Saturday — 1000 — last supernatant from ① (Electroporator)
— 60 hrs p.e.

9/11/94 Sunday — Electroporation

10 µl LSN 9/7/94 + 10 µl LSN variant 9/9/94 + 20 µl PI-5 8/21/9
1.65 kV ; 10" RT p.e
1÷2 x 10⁷



9/12/94

9/12/94
Monday

TF1.8 cells electroporated 9/11/94 a-Lassa (~10%)
a-VEE (~10%)

after infection — 5.22 · 10 particles/ml
practically, no VEE antigen
(no cpe found)

(13502)
8133
5369

56°C pV3000 1) BstEII
2) SpeI

BstEII:

10 × (2)	25
pV3000 DNA	50
H2O	175
Enzyme	5 (8 u/µl)
	56°C

SpeI

10 × (3)	25
0.1% BSA	25
H2O	170
pV3000 DNA	50
Enzyme	3 (8 u/µl)
	56°C

pV3000
BstEII
SpeI

16[15] ÷ 18[30]

38

9/13/94
Tuesday

Transcriptions

LSN - mMmM (Ambion)

water	9
10x buf	4
2x NTP/cap	20
GTP 30mM	2
DNA/NotI	0.7
U3 ATP	1
10x Enz	4
	40µl

4 hrs (!) - wrong ?!

P1-5 (Promega)

water	17
5x buf	20
100mM ATP	7.5
100mM UTP	7.5
100mM CTP	7.5
100mM GTP	4.6
25mM cap (Pharm)	24
DNA/NotI	4
U3 ATP	1
10x Enzyme	10
	100µl

m7G(5')ppp(5')G
27-4635-02 25 u
Lot 4054635021
For in vitro use only
Store at -20°C
Made in USA
Pharmacia Biotech

6%
54%

Nothing!

No change

Electroporation

16 µg PI-5 8/21
16 µg LSN 9/9 variant
1.7 kV ; 10' RT

~~80-100%~~ 80-90% LSN
50-60% PI-5

9/14/94
Wednesday

supernatant: probes:

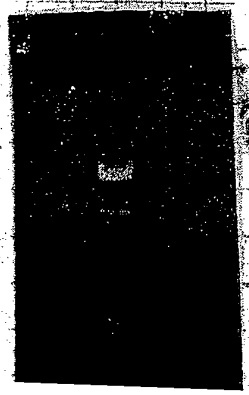
4.30	8:30 am	~~14~~ hrs p.e.	✓ (14) hrs p.e
12:30	~~19~~ 18 :30 am	~~18~~ hrs p.e	✓ (18)
16:30	~~16~~ :30	22 hrs	✓ (22)
24:30	~~20~~ :30	30 hrs	✓ (26)
5	~~10~~ :30		✓ ~~30~~ (30)
5	8:00		(37.5)
5	15:00		(44.5)
6	15:00 transcription		(6 ~~2~~ hrs p.e

LSN

water	8.9 µl
~~5~~ x but	8 µl
25mM NTP/-G	12 µl
25mM cap	4.8 µl
DNA/NotI	0.7
H3 ATP	1
10 x Buf G 100mM	4
	~~0.6~~ µl
	40 µl

PI-5

water	~~1~~ 4
5 x but	8
25mM NTP/-G	12
25mM cap	9
DNA/Not	4
H3 ATP	1
10 Buf 2	4
G 100mM	~~2.5~~
	40 µl



LSN

PI-5 OK

LSN washed (2.5)%

PI-5 washed 2.1%

5 µg/20 µl
8 µg/40 µl

A 053

9/14/94
Wed

supernatant: probes:

8:30 am — 14 hrs p.e. ✓(14) hrs p.e
10:30 am — 18 hrs p.e ✓(18)
12:30 — 22 hrs ✓(22)
14:30 — 26 hrs ✓(26)
16:30 — 30 hrs ✓(30)
18:30 (37.5)
20:00 (44.5)
22:00 (6 hrs p.e)
transcriptions

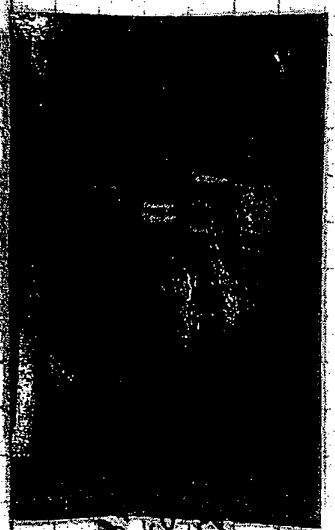
LSN

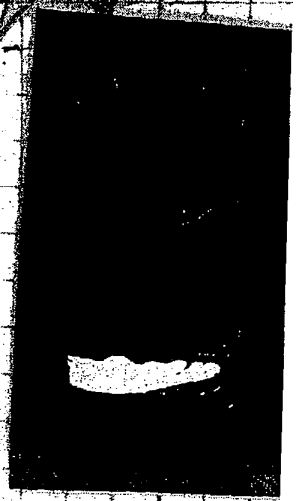
water	8.9 µl	
5x but	8 µl	
25mM NTP/-G	12 µl	
25mM cap	4.8 µl	
DNA/NotI	0.7	
U3 ATP	1	
10x Buf	4	
G 100mM	0.6 µl	
	40µl	

P1-5

water	14
5x but	8
25mM NTP/-G	12
25mM cap	9
DNA/Not	4
U3 ATP	1
10 Buf	4
G 100mM	0.5
	40µl

5µg/20 8µg/40





LSN
P1-5 OK
LSN washed 2.5%
P1-5 washed 2.1%

Mw band

EcoRI pVS000

Dba EcoRI cauta!

Transcription

	pV3000
water	8.9
5× buf	8
25mM NTP's-A	12
25mM cap	4.8
DNA/Not I	0.7
H³ ATP	1
10× Buf	4
100mM GTP	0.6
	40 µl

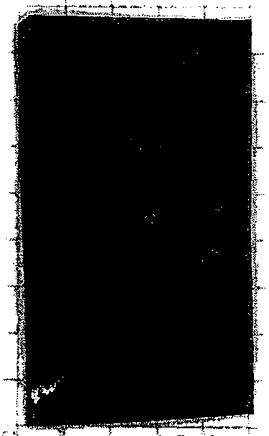
9/15/94

0.95%

RNA

pV3000 pV3000 M - RNA ladder BRL

+DNAse



pV3000 KpnI

7µl on gel

(P2) 14µl water

2 µl pV3000/Kp

2 µl Lig Buf +

2 µl T4 DNA

O/n 4°C

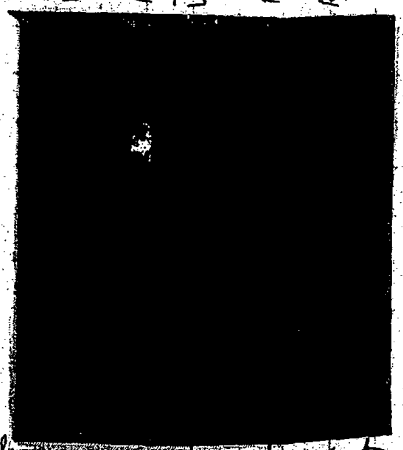
(10)µl/50µl of c

B

Transcriptions

	pV3000 OK		pT-5 OK	
water	7.9	(5.8)	6	(3.9)
5 x Buf	8	8	8	8
25 mM NTP/G	12	12	12	12
25 mM cap	4.8	(6.4)	7.2	(8.4)
DNA/Not I	1.7	(2)	1.2	(2)
α 3 ATP	1	1	1	1
10 x Enz	4	4	4	4
100 mM GTP	0.6	(0.8)	0.6	(0.7)
	40 µl		40 µl	

1868.0



~~0.6%~~
0.62%

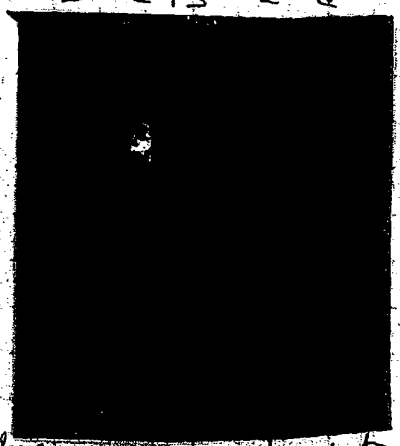
1.37%

T4 polymerase treatment

	Apa I / ~~Sph I~~	pV3000	Apa I / ~~Sph I~~
water	40		20
DNA/Enz	3		25
10 x Buf	5		5
100 mM dNTP	0.5		0.5
T4 (P)	1		1
	50		50

RT 20' + 150 µl water
+ 200 µl phenol-chl
+ tRNA.
+ NaCl
+ EtOH conc. 1 2n'a

5 M NH_4Ac	12	7.5
1 M cap	4.8 (6.4)	7.2 (8.4)
DNA/Not I	1.7 (2)	1.2 (2)
0.5 ATP	1	1
10 x Buf	4	4
100 M M GTP	0.6 (0.8)	0.6 (0.7)
	40 Ml	40 µl



1868.0

1516

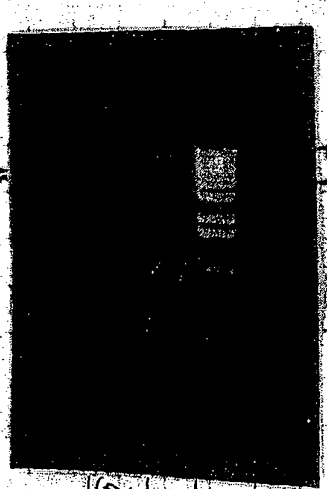
~~0.8%~~
0.62%

1.37%

T4 polymerase treatment

Apa I/~~Bgl II~~ (pV3000)

water	40
DNA/Buf	3
10 x Buf	5
100 mM dNTP	0.5
T4 (P)	.1
	50



Apa ~~I/Bgl II~~

	20
	25
	5
	0.5
	1
	50

RT 20' + 150 µl wat
+ 200 µl phenol-cl
+ tRNA
+ NaCl
+ EtOH 500 µl 20'

07/16/94
Friday

1) checking yesterday's transcription

24.71

2.7% = 2%

V3000 : 343.810

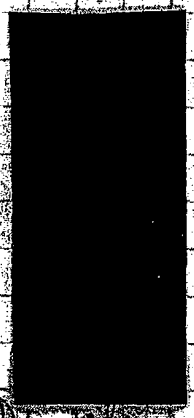
3.4% = 3%

P1-5 : 7[illegible]

V3000 : 2 ÷ 2.5 µg/10 µl

P1-5 : 3 - 3.4 µg/10 µl

2) cleavage KpnI + ⑥ React + 0.01% BSA



10 µl on the gel

Ligation:
30 min
37°C
O/N at 65° → -70°C

2 µl of ⑩
15 µl of water
2 µl ~~[illegible]~~ Ligase buff
1 µl Ligase (T4)

3) clones from P2: (K)/100 ∞
(P2)/100 ∞
(P2)/100 many

~~KpnI : [illegible]~~ ; 65°C 95' ;
cut 2)

4) IFA for a-lassa (of BHK infected w/ kpnI cut pGEM + P1-5)

IFA: 14 hrs post infection (CSN + P1-5)
a-lassa # 288 conjugate CSN + P1-5 helper

lassa (infection) IFA 9/16	0.8 · 10⁵/ml	1.1 · 10⁵/ml	1.5 · 10⁵/ml	3.1 · 10⁵/ml
	14 hrs p.e.	18 hrs p.e.	22 hrs p.e.	26 hrs p.e.
14 hrs post infection	2.6 · 10⁵/ml	1.4 · 10⁵/ml	0.4 · 10⁵/ml	not detected 110 (negative control) not infected BHK cells
	30 hrs p.e.	37 hrs p.e.	44 hrs p.e.	

V3000 : 2÷2.7 µg/10 µl
P1-5 : 3-3.4 µg/10 µl

A 058

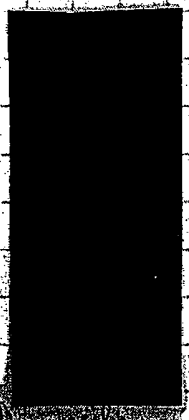
2.7% = 2%

3.4% = 3%

V3000

P1-5

2. cleavage KpnI + ⑨ React + 0.01% BSA



10 µl on the gel

Ligation: 2 µl of ⑩
30 min 15 µl of water
37°C 2 µl ~~Ligase buf~~ Ligase buf
W° at 65° → -70°C 1 µl Ligase ⑦

from P2: ⑧/1000 ∞
P2/900 ∞
P2/100 many

KpnI : рестриктаза ; 65°C 95° ;
~~генетическая~~ ;
сеч. 2)

4) IFA for a-lassa (of BHK infected w/ kpnI cleaved particles)
IFA: 14 hrs post infection (CSN + P1-5)
a-lassa # 288 conjugate LSN + P1-5 helper

lassa (infection) IFA 9/16	$0.8 \cdot 10^5$/ml 14 hrs p.e.	$1.1 \cdot 10^5$/ml 18 hrs p.e.	$1.5 \cdot 10^5$/ml 22 hrs p.e.	$3.1 \cdot 10^5$/ml 26 hrs p.e.
14 hrs post infection	$2.6 \cdot 10^5$/ml 30 hrs p.e.	$1.4 \cdot 10^5$/ml 37 hrs p.e.	$0.4 \cdot 10^5$/ml 44 hrs p.e.	not detected 110 (negative control) not infected BHK cells

amount of particles/ml of supernatant of BHK cells transfected with LSN segment + P1-5 helper

Transfection efficiency was estimated as 80-90% / 50%

9/13/44

BHK cells were subconfluent before infection

Compare to 9/
5.22 10^5/ml
when efficiency was
100% for both segments

IFA: (10 hrs) p.i. LSN replicon PL-5 helper

1:1 Sca dilution 9/16	$3 \cdot 10^8$/ml 14 hrs p.e	$1 \cdot 10^5$/ml 18 h	$1.4 \cdot 10^5$/ml 22 hrs p.e	$3.6 \cdot 10^5$/ml 26 hrs p.e.
	$1 \cdot 10^5$/ml 30 hrs p.e.	$2.8 \cdot 10^5$/ml 37 hrs p.e	$6.4 \cdot 10^5$/ml 44 hrs p.e	(nc) "0"

$0.5 \cdot 10^5$/ml
68 hrs p.e
see 9/20 IFA

Precipitation and cleavage w/ Avr2 of
P3 and P4
ΔApaI/BstEII ΔApaI/PlI

20 µl + 80 µl water
+ 100 µl phenol+chloroform
sup + ~~70~~ µl NaCl 1M
+ 1 µl 10 mg/ml tRNA
+ 300 µl EtOH
-70°C 20' spin, wash 2x 75% EtOH, dry, discuss
in ~~15~~ µl water + 2 µl React(2) + 1 µl Avr II

transformation into DH5α (max efficiency)

Do not forget to check the same supernatants (14; 18; 22; 26; 30; 37; and 44 hrs p.e.) undiluted, for VEE protein! LSN+PL-5 replicon + helper

9/18/94
Sunday

T4 polymerase

	Apa HindIII	Apa SpeI
DNA	45 µl	10 µl
10x T4 buf	5 µl	5
100 mM dNTP	0.5 µl	0.5
T4 (P)	1	1
water		35
	50	50

20' RT

+150 µl water + 200 µl phenol-chloroform,
1' vortex 5' spinning
+ 30 µl 1M NaCl + 2 µl tRNA 10 mg/ml
+ 600 µl EtOH 30' -70 °C
dissolve in 10 µl H2O

4 µl → gel

Electroporation

1	LSN	10 µl 9/9	5 mg/10 µl variant	µV 300	30 µl 9/15 2÷2.7 µg/10 µl
2	LSN	10 µl 9/14	2-2.5 µg/10 µl	µV 300	1.7 kV 10' at RT p.e.
3	LSN				

18 30

4 µl + 10 µl water
2 µl ligase buffer
+ 2 µl T4 DNA ligase

LSN + pV3000 as a helper

14 hrs p.e.		
8 ow	–	14 hr p.e.
12 ow	–	~~18~~ hrs p.e
16 ow	–	22 hr p.e.
20 ow	–	26 hrs p.e
22 ow	–	30 hrs p.e.
9 ow	–	38 hrs p.e
15 ow	–	45 hrs p.e

a - Cassa (~ 60 - 80% ; up to 90%)
a - VEE (~ 50 - 70% ; maybe 60-70%)

(14 hrs p.e.)

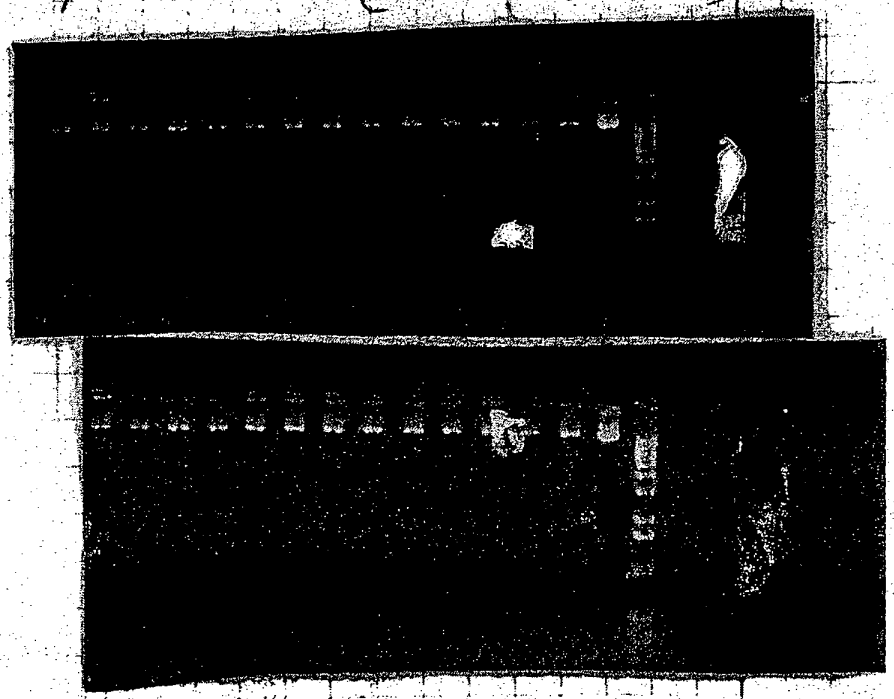
a - Cassa (~ 60 - 80% ; ... 90%?)
a - VEE (60 - 70%)

(40 hrs p.e.)

P2 miniprep (V3000 Δ KpnI) No. 1 ÷ 16
STET - LSTET method

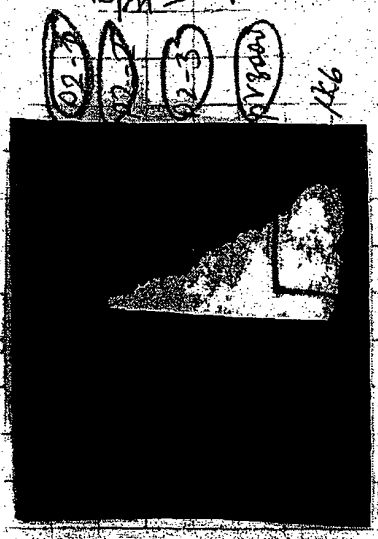
cleaved KpnI

dissolved in 50 µl of water { 1:10 10x React (4) / 1:10 0.1% BSA }
2 µl of DNA + 50 µl Rec. Mix

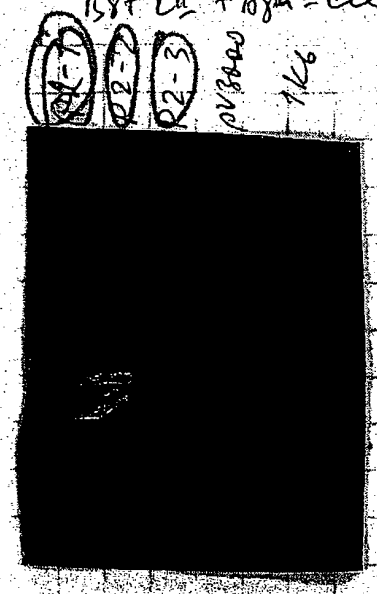


9/20/1994
Tuesday

Kpn I + ApaI cleavage



BstEII + ApaI cleavage



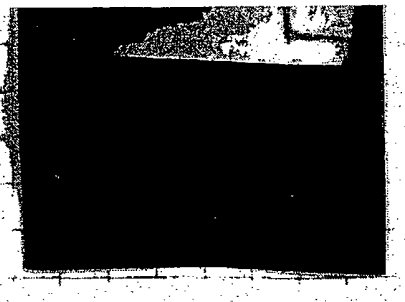
(14) & (15) in "replicon" box at -70°C freezer
there are frozen E. coli cultures
of ΔBstEII – ApaI } of mutant "repair" genes
ΔSpeI – ApaI } helpers

Saccharomyces 2mL - LB cultures of:
1. P2-1
2. 14 } makes SpeI
3. 15 } BstEII

P2-1

ΔKpnI pV3000 helper

8:30-10:30 Infection with recombinant particles and
18:30 IFA LSN + pV3000 (particles)



(14) & (15) in "replicon" box at -70°C freezer
these are frozen E. coli cultures
of Δ BstEII – BspaI } of "mutant" ~~repair~~ genes
Δ SpeI – BspaI } helpers

A 063

3 aliquots 2 ml - LB cultures of:
1. P2-1
2. 14 } Mike's SpeI
3. 15 } BstEII

P2-1

Δ KpnI pV3000 helper

8:30 - 10:30 Infection with recombinant particles and
18:30 IFA LSN + pV3000 (particles)
(8-10 hrs p.i.) (8) hrs p.i.

10^{-3} dilution of supernatant

47

LSN + pV800 RNAs transfected, cups collected

↓

10-3 dilutions of supernatants

100 µl of diluted sups

2 hrs infection at +37°C

+ 300 µl medium incubation continued at 37°C

9/20 8 hrs p.i. (+10 hrs post infection)	0 14 hrs p.e.	0 18 hrs p.e.	0 22 hrs p.e.	1(+) Kreika 0.1·10⁵/ml 26 hrs p.e. ←
	0.3·10⁵/ml 30 hrs p.e.	0 38 hrs p.e.	0.5·10⁵/l 68 hr. see 9/	0 "NC" + BHK + medium, supernatant

a - Lassa #288 conj.

LSN+P1-5

Basically, same, IFEA was done 8 or 24 hrs post infection

LSN + pV800 RNAs

~24 hrs post infection	0	nd	nd	0
	3·10⁴/ml	nd	6·10⁴/ml	nt "nc"

a - Lassa #288 conj. LSN+P1-5

9/21/94 Wednesday

8 hrs p.i.	nd	nd	nd	nd
	2·10⁵/ml	nd	nd	nd

a - VEE

a - mouse conj.

~~titer must be tested~~ ~~titer must be~~ test titer must be determined

TRANSCRIPTIONS

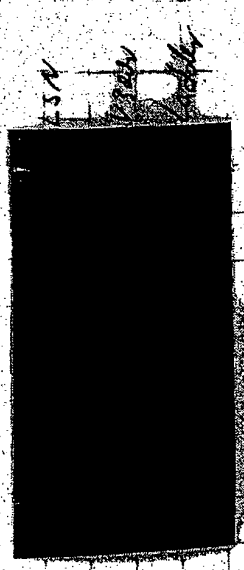
	LSA	V8000
water	8.8	~~7.8~~ 7.4
5x Buf	8	8
25mM ATP/-G	12	12
25mM Cap	4.8	~~8.3~~ 4.8
20u/ul RNTI	0.7	2
4.3 DTT	1	1
GTP 100mM	0.7	0.8
10x Enz	4	4
	Cap/G 1.7:1	1.5:1

LIGATIONS

	P3 BssT (~~4800~~ 2400 ~~0.85~~ EII-Apa I)	P4 SpeI (~~2800~~ 1400 SpeI-A
Water	10	10
DNA/RNA (P)	6	6
10x Ligase	2	2
~~Pf~~ DNA Ligase	2	2
	20 µl	20 µl

9/22/94
Thursday

1) PM : 2582



5' / Cassq

6' / V3000

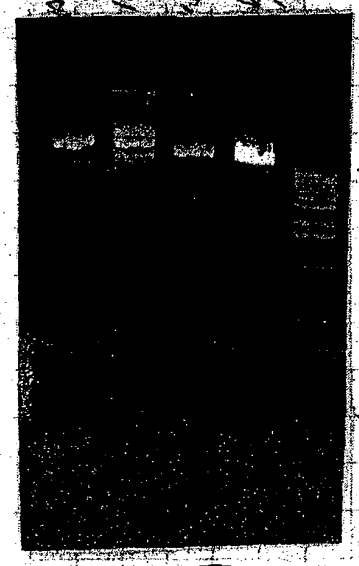
Transcriptions:

(SN can be used
(~ 0.5 µg/µl ⇒ 15 µl per electroporation)

V3000 – bad quality RNA,
pV3000/NotI is (twice) phenol
treated and precipitated

2) Transformation P3 (BsteII Spe I/pV3000)
P4 (Δ Spe I Spe I/pV3000)

3) DNA isolation P2-1
14
15 } Mike's helper



degradation of DNA ??

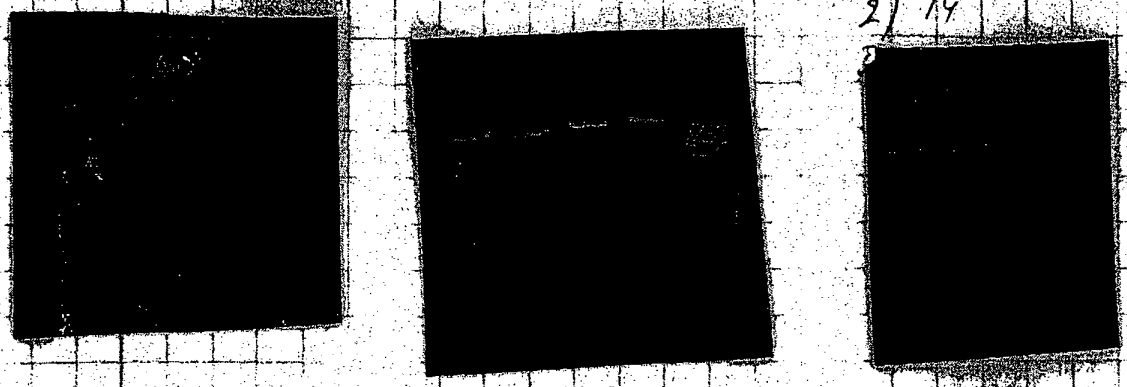
4/1 Alonzo Not I 1/10 analytical

50

7/23/94

P4 & Spe I Apa / V3000 (2) clones
T4 (P)
T4 (E)

Preparative Cleavage Not I 1) P2-1
2) 14



V3000 / 6 Apa I Spe I / T4 and } purified w/ Wizard
V3000 / 6 Apa I Sst II / T4 } PCR prep kit

CREATION

	P3/Bst	P4/Spe
water	[redacted]	25
10 x A	45	20
10 x L	5	5
Lipase	1.25	1.5
	50 µl	50 µl

30' at 37°C

50 µl → E. Coli DH5α Transformation

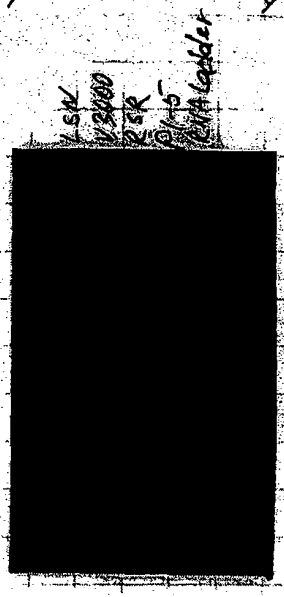
P3/Bst : 33 clones } per 200 µl / plate
P4/Spe : none

51

9/24/94
Saturday

TRANSCRIPTIONS OK

	LSN	V3000	RSR	PI-5
water	8.8	8.8	3.9	3.9
5× buf	8	8	8	8
25 mM NTP/-G	12	12	12	12
25 mM cap	4.8	4.8	8.4	8.4
RNA/NOFI	0.7	0.7	2	2
α³² ATP	0.9	0.9	0.9	0.9
GTP 100 mM	0.7	0.8	0.7	0.7
10× Enz	4	4	4	4



LSN { 3.8%

V3000 { 2.9%

RSR { 4.25%

PI-5 { 3.1%

5/94
unday

ELECTROPORATION 20:00

1.	V3000	16 µl 9/24 + 10 µl 9/24	NO
2.	LSN + V3000	— / —	+ 10µl 9/24 + 7µl 9/24
3.	LSN + RSR	10 + 7 Lassa + RNA RSR	15µl 9/24
4.	LSN + P1-5	10 + 7 Lassa + RNA P1-5	20µl 9/24

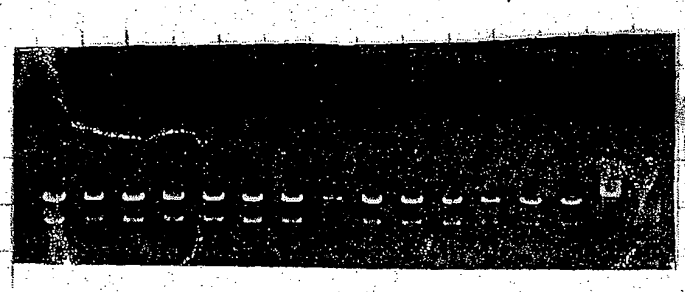
2.9% 0.9%

0	V3000 :	16µl 9/24 + 10 µl 9/24	
LSN	LSN + V3000 :	16µl (2.9%) 9/24 +	10µl 9/24 (0.9%) V3000 & 10µl (3.8%) 9/24 +
	LSN + RSR	15µl (4.25%) 9/24	— / —
	LSN + RSR	20µl (3.1%) 9/24	— / —

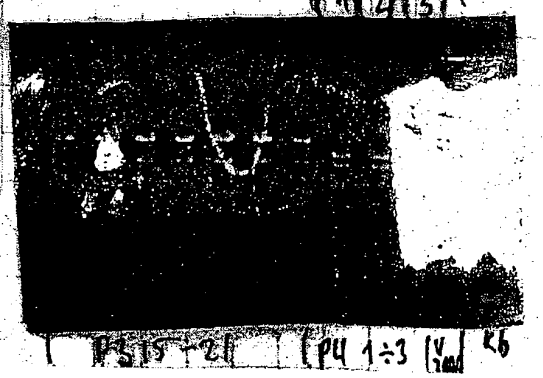
5.54 µl
5.54 µl + 7.3 µl LSN
5.38 µl + 7.3 LSN
6.2 µl + 7.3 LSN

9/26/94

	8:00	(12 hr p.e.)	0.5 ml sup. samples taken
	12:00	(16 hr p.e.)	— / —
	16:00	(20 hr p.e.)	— / —
	19:20 ~~20:00~~	(24 hr p.e.)	— / —
	~~24:00~~	(30 hr p.e.)	— / —
27	8.00 8.00	(38 hr p.e.)	—
27	14.30	(46 hr p.e.)	
	19.30	(~ 51 hr p.e.)	
28	8.30	~ 64 hr p.e	
29	8.20	(~ 88 hr p.e)	
30	8.20	Mini Preps P4/1 ÷ 3	Cleavage
		(~ 112 hrs p.e) P3/1 ÷ 21	



P3 1÷14



P3 15÷21 P4 1÷3

58

IFA a-lasse

① "0" ② 50÷70%

50÷70% 50÷70%

③ ④

0.1ml

ml PBS
9/89

a-VEE

① 40÷50% ② 40÷50%

40÷50% ④ 40÷50%

③

TRANSCRIPTIONS

① LSN

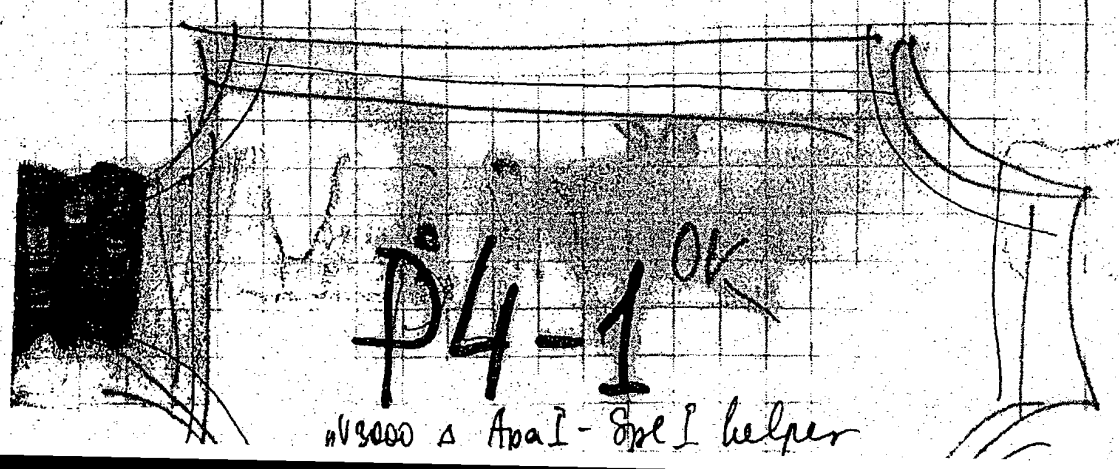
② pV3000 as in 9/24

LSN

V3000

LSN 4.4%

V3000 2.2%



P4-1 OK

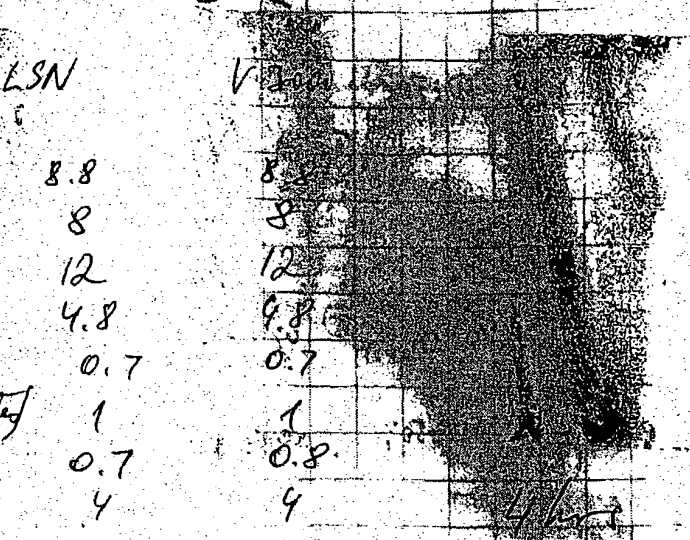
pV3000 Δ AvaI - SpeI helper

54

9/27/94
Tuesday

TRANSCRIPTIONS OK

	LSN	V
water	8.8	8.8
5x Buf	8	8
25 mM NTP/-G	12	12
25 mM cap	4.8	4.8
SmaI/NotI	0.7	0.7
H^3 CTP (1:1 ethanol/water)	1	1
100 mM GDP	0.7	0.8
10x Enzmix	4	4



Lysate at 37°C

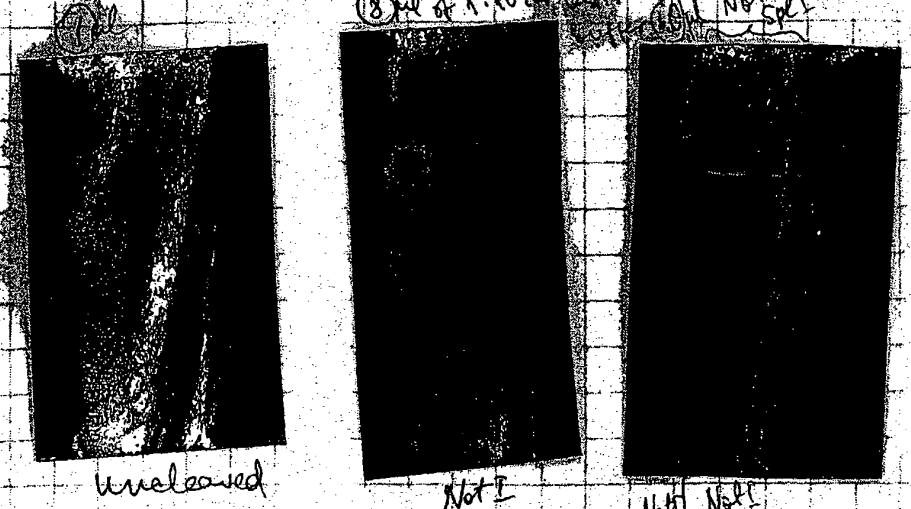
9/28/94
Wednesday

5.78%

OK

55

P4-1 helper (Δ SpeI - SpeI) isolation



Not I SpeI no cleavage (17°C 2h)
SpeI does not exist!!!
Δ SpeI Δ SpeI!!!

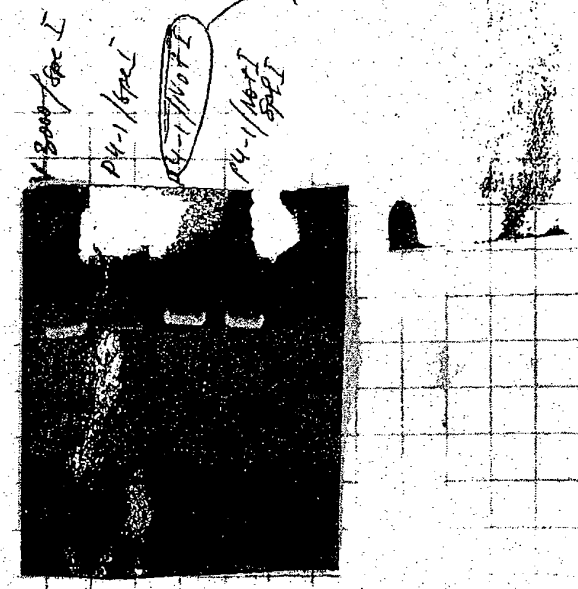
9/29/94
Thursday

P4-1 Not I precipitation, dissolved in 50 µl water

1 µl - 1% gel

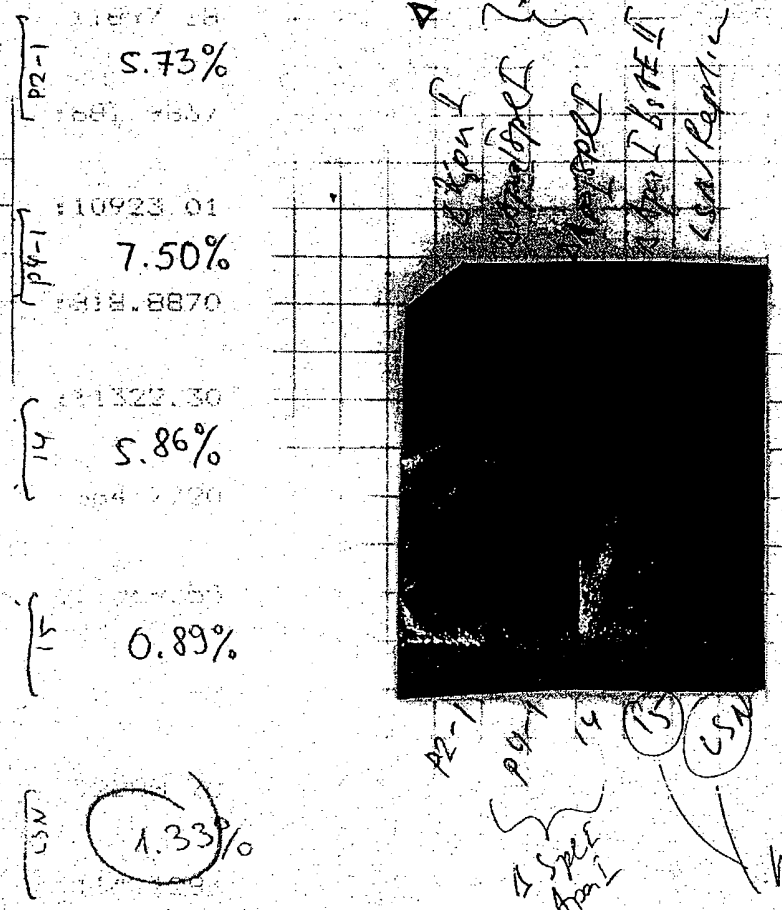
→ for transcriptions

9/30/94
Friday



TRANSCRIPTIONS

	P2-1	P4-1	14	15	CSN
water	9.1	8.3	8.3	8.3	8.8
5x Buf	8	8	8	8	8
25 mM NTP-G	12	12	12	12	12
25 mM cap	4.8	5.6	5.6	5.6	4.8
DNA/Not I	1	1	1	1	0.7
H3 UTP	1	1	1	1	1
100 mM GTP	0.7	0.7	0.7	0.7	0.7
10x Enzyme	4	4	4	4	4
	1:1.7	1:2	1:2	1:2	1:1.7



P2-1 5.73%

P4-1 7.50%

14 5.86%

15 0.89%

CSN 1.33%

10/1/1994

Bjk cells are ~ 70% confluent

10/2/94

ELECTROPORATION $2 \cdot 10^7$ cells/ml 1.500 kV
~10,000,000 cells/cuvette

(1) NC (NO RNA) 12.00 I

(2) LSN + P2-1 (5 kpm I)

LSN: 1.7 µl of (3.8%) ⇒ 0.646 µg
10 µl of (2.5%) ⇒ 2.5 µg
6 µl of (5.78%) ⇒ 3.468 µl
+ 6.614 µg
~~total~~

P2-1: 12 µl of (5.73%) ⇒ + 6.876 µg
~~total~~ II

(3) P2-1 12 µl of (5.73%) ⇒ + 6.876 µg
~~total~~

(4) LSN 1.7 µl (3.8%) ⇒ 0.646 µg
10 µl (2.5%) ⇒ 2.5 µg
6 µl (5.78%) ⇒ 3.468 µg
+ 6.614 µg
~~total~~

(5) LSN + V3000

LSN: 1.7 µl of (3.8%) ⇒ 0.646 µg
6 µl of (2.5%) ⇒ 1.5 µg
8 µl of (5.78%) ⇒ 4.624 µg
+ 6.77 µg ~~total~~ III

V3000: 2.5 µl (2.7%) ⇒ 0.675 µg
2 µl (2.9%) ⇒ 0.58 µg
10 µl (4.88%) ⇒ 4.88 µg
+ 6.135 µg ~~total~~

LSN: 1.7 μl of (3.8%) ⇒ 0.646 μg
10 μl of (2.5%) ⇒ 2.5 μg
6 μl of (5.78%) ⇒ 3.468 μl
+ 6.614 μg
~~total~~

P2-1: 12 μl of (5.73%) ⇒ + 6.876 μg
~~total~~

(3) P2-1 12 μl of (5.73%) ⇒ + 6.876 μg
~~total~~

(4) LSN 1.7 μl (3.8%) ⇒ 0.646 μg
10 μl (2.5%) ⇒ 2.5 μg
6 μl (5.78%) ⇒ 3.468 μg
+ 6.614 μg
~~total~~

(5) LSN + V3000

LSN: 1.7 μl of (3.8%) ⇒ 0.646 μg
6 μl of (2.5%) ⇒ 1.5 μg
8 μl of (5.78%) ⇒ 4.624 μg
+ 6.77 μg ~~total~~

V3000: 2.5 μl (2.7%) ⇒ 0.675 μg
2 μl (2.9%) ⇒ 0.58 μg
10 μl (4.88%) ⇒ 4.88 μg
+ 6.135 μg ~~total~~

(6) V3000

2.5 μl (2.7%) ⇒ 0.675
2 μl (2.9%) ⇒ 0.58
10 μl (4.88%) ⇒ 4.88 / 6.135 μg + = ~~total~~

58

Supernatant Samples

16[00]

20:20

00:

1994 when IFA was done 6:

10

TFA a-La

18hrs pre

Coolpack study

(1)

(4)

LSN

LSN-larva replican

P2-1 - ΔKpnI V2000 helper

a-VEE

P2-1 doesn't work

dilutions

VEE 1:25

a-mouse 1:25

(1) NC 0

(2) LSN P2-1 high background "0"

(3) P2-1 0

(4) LSN

(5) high background LSN V2000 20-40%

(6) V2000 20-40%

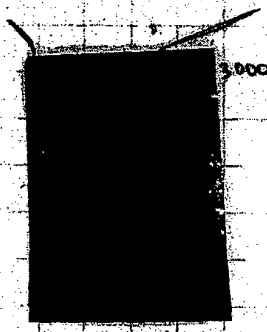
Spec

Not linear

pV2000:	6305;	7200;	13502
ΔKpnI p2-1:	12098		12048
6 ApaI SpeI p4-1:	2754;	7200	9951
14:	2754;	7200	9951
15:	7679		7679
P1-5:	6513		6513
RSR:	6513		6513

A 077

cleaned DNA template
~~transcriptions~~



3000/Dat1 (partial)

BUK - 38 pass.

10/5/1994 Wednesday

TFAs of supernatants from electroporation 10/2/94
(see previous page, p. 60)

pV3000 - clat II in Boehringer - Mannheim (A) buffer

Act II
partial

10/6/1994

Electroporation 14:00 ~ $2 \cdot 10^7$ cells
as in

Samples:

1) 7.728 µg SN 5µl of 0.5 µg/µl + 6 µl
8.28 µg P1-5 17 µl of 0.34 µg/µl + 6 µl — 4µl of 0.44 µg/µ
— µl of 0.14 µg/µ

2) 7.728 µg SN as in 1)
8.0 µg SL 20 µl of 0.4 µg/µl

3) 8.28 µg P1-5 as in 1)

4) 6.53 µg SN 5µl of 0.5 µg/µl + 2.5 µl of 0.578 µg/µl + 6 µl 0.44 µg/µ
6.83 µg 3000 14 µl of 0.488 µg/µl

P5

pV3000 cleavage with Cat II

Water	160 µl
10x React 2	20 µl
pV3000	10 µl
Cat II	3 µl

at 37°C 2:5 [illegible]

17/9/1994

[redacted]

No cleavage 0/12 (a 20 hours)!!!

a-lassa

(2) LSN P2-1 — 10⁻³ dilution — 4hrs 8 12 18 — 22hrs 26hrs 30hrs 46hrs — 10 10⁷ 10⁸ 10

(3) LSN V3000 — 10⁻³ dilution — 4 8 12 18 — 22 26 30 41 — 4·10⁶ 10⁷ 10⁸ 10⁷

a-VEE ZABRAT

(5) LSN V3000 — 10⁻³ dilution — 4 8 12 18 — 22 26 30 46 — 10⁷ 10⁷⁻⁸ 10⁷⁻⁸ 10⁷

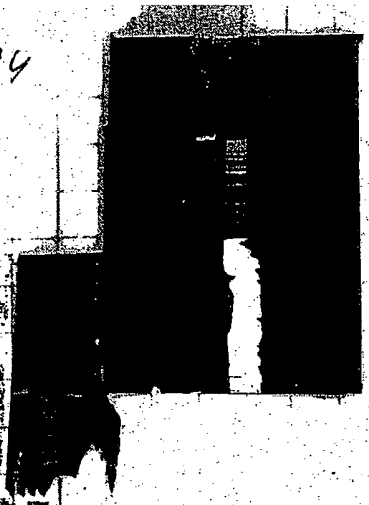
(6) V3000 — 10⁻³ dilution — 4 8 12 18 — 22 26 30 41 — 10⁷ 10⁷⁻⁸ 10⁷⁻⁸ 10⁷

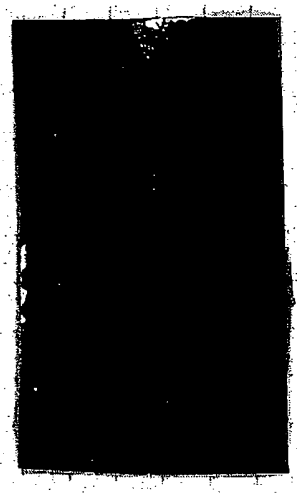
Control

(2) LSN P21 — 10⁻³ dilution — 4 8 12 18 — 22 26 30 46 — 0 0 0 0

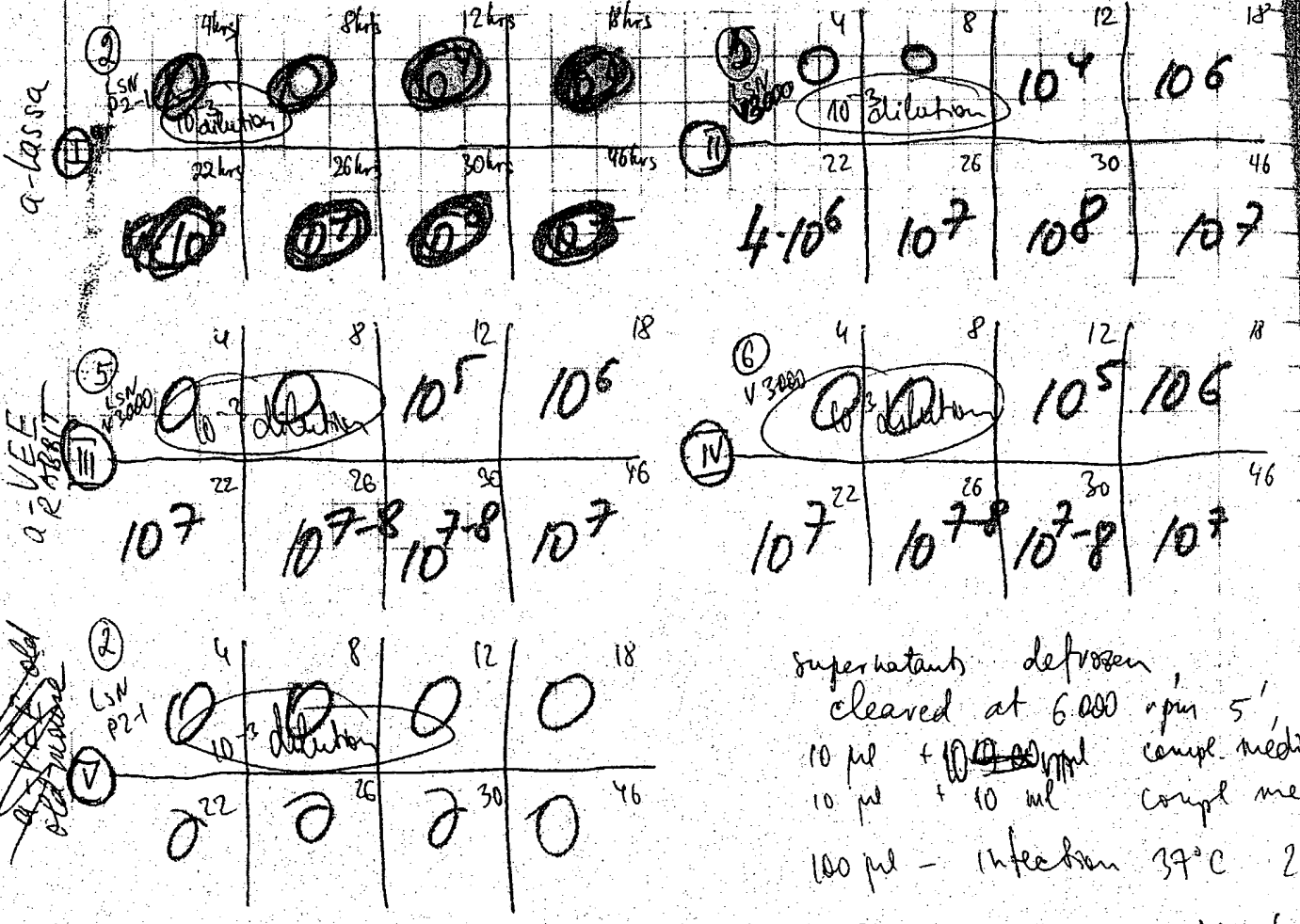
supernatants defrozen
cleared at 6.000 rpm 5'
10 µl + 10 ml complt m
10 µl + 10 ml complt m
100 µl — [illegible]

/1494





No cleavage
o/n (~20hrs)!!!



supernatants defrozen
cleared at 6.000 rpm 5'
10 µl + 10 ml compl. médium
10 µl + 10 ml compl med
100 µl - infection 37°C 2h
+ 300 µl compl. medium

Supernatant samples

18[00] (hrs. p.e.)

22[00] (p.e.)

1[30] (11.5 hr. p.e.)

7/94 8[00] (18 hr. p.e.)

12[00] (22 hrs p.e.)

16.30 (26.5 hrs)

~~19:30~~ (29.5 hrs)

24[00] (34 hrs)

8/94 9[30] (43.5 hrs)

9/94 14.00 (72 hrs)

11/94 19:00 (120 hrs)

air bubbles (removed) processing heating 12?

① 15 μg RNA CSN+P1-5 medium pink, much fewer cells than in ②

② 15.7 μg RNA CSN+RSR medium yellow, many cells

③ 8.3 μg RNA P1-5 yellow

④ 13.4 μg RNA CSN+V3000 yellow

① is still pink ②③④ - yellow but quantity of cells attached to surface is about the same in all four tubes

media color is about the same intensity of cells attached is about the same, ~~only~~ ① has much greater # of attached cells, ② a little bit less than in the ~~media~~ and number of cells about the same in all 4 samples

18 hrs p.e. CAT2 slides:

EEA in cassette — a-VEE

① ~80% ② ~60% ① 20-30% ② ~30-40%

③ 50% ④ ~30% ③ 0% ④ ~40%

efficiency of transfection ~~was~~ also ~ 30% in cassette for VEE antigen

10/7/94 TRANSCRIPTIONS

	CSN	V3000	P2-1	P3
water	18.6	8.8	8.3	7.7
5x buf	16	8	8	8
25mM NTP/-G	24	12	12	12
25mM Cap	9.6	4.8	5	5.6
RNA/NOT C	1.5	0.7	1	1
U3UTP	1	1	1	1
100mM GTP	1.4	0.8	0.7	0.7
10x Enzmix	8	4	4	4

(..5·10⁴)
LSN undiluted 1:10 (NC)

4	8	12	18
22	26	30	36

46	VEE 10^{-2} 30 hrs NC	many false-positive, probably because of some cross-reactivity with lassa with VEE 3.5 slides
80		

lassa

Infection with supernatants from electroporation 10/2/94

a-lassa

③ LSN + V3000; dil. 10^{-4} → dilution of supernatant

12 hr/10^{-4}	18 hr/10^{-4}	22 hr/10^{-4}	26 hr/10^{-4}
wt	wt	wt	wt
30 hr/10^{-4}	34 hr/10^{-4}	44 hr/10^{-4}	80 hr/10^{-4}
~0.10^5	2.10^5	3.10^5	3.10^5 particles/ml

(II) ④ LSN; dil. 1:10 (10^{-1}); NC

4 hr/10^{-1}	8 hr	12 hr	18 hr
0	0	0	0
22 hr	26 hr	30 hr	34 hr
0	0	0	0

(III)

(IV)

⑤ LSN + V3000; dilution 10^{-2}

4 hr/10^{-2}	8 hr	12 hr	18 hr
~1.10^3	~1.10^3	$2.3.10^3$	$7.8.10^3$
22 hr	26 hr	44 hr	10 hr
$3.5.10^4$	$3.5.10^4$	$2.2.10^5$	$2.1.10^5$
$0.4.10^5$	⑥ LSN (continued); dil. 10^{-1}; NC		
44 hr	V3000 (sample ⑤) at 10^{-2} dilution		
0			
80 hr			
0			

many false-positive, probably because of some cross-reactivity with VEE antigens of a-lassa-conjugate

Meas. I

Meas. II

1.46% = 1.51%

:19299.45
1.57% = 2.15%
:304.3364 :363.4319

:1874.55
0.4%
:74.44427

0.2%

① LSN + V3000

~~⑥ V3000~~

12 ~~2 x 2 samples~~ ~~2 samples~~

4 hr	18 hr	22 ~~23~~	26 ~~25~~ equivalent
80	36	46	80 − 4
~600	~1000	~800	~~400~~
300	at higher dilutions		
~~0~~ · 10^5	$2 \cdot 10^5$	$5 \cdot 10^5$	$3 \cdot 10^5$

4	8	12	18
~$1 \cdot 10^3$	~$1 \cdot 10^3$	$1.3 \cdot 10^2$	$8.7 \cdot 10^3$
22	26	46	80
~~$5 \cdot 10^4$~~	~~$5 \cdot 10^4$~~	$3 \cdot 10^5$	~~$4.8 \cdot 10^{-3}$~~

supernatants from operation 10/2/94

⑤ LSN + V3000; dilution 10^{-2}

4 hr / 10^{-2}	8 hr	12 hr	18 hr
~$1 \cdot 10^3$	~$1 \cdot 10^3$	$2 \div 3 \cdot 10^3$	$7 \cdot 8 \cdot 10^3$
22 hr	26 hr	46 hr	80 hr
$3.5 \cdot 10^4$	$3.5 \cdot 10^4$	$2.2 \cdot 10^5$	$2.1 \cdot 10^5$

$4 \cdot 10^5$

② LSN (continued); dil. 10^{-2}, MC

44 hr	V3000 (sample ⑥) at 10^{-2} dilution 30 hrs p.e.		
0			
80 hr			
0	empty		

many false-positive, probably because of some cross-reactivity with VIP epitopes of α-lasa-conjugate

6

9 – VEE

⑤ LSN + V3000 ; dil 10^{-4}

V

12hr	18hr	22	26
less	less	less	less
30	34	44	80 hr p.e.
~10^6–10^7	10^7–10^8	10^7–10^8	~10^7

⑥ V3000 ; dil 10^{-4}

VI

12	18	22	26
less	less	less	less
30	34	44	80 hr p.e.
less	10^7–10^8	10^7–10^8	less

④ LSN ; dil. 10^{-1} ; NC

IX

12	18	22	26
practically			
30	36	46	80 hr p.e.
nothing			

⑤ LSN + V3000 ; dil 10^{-2}

VII

4	8	12	18
22		44	80 hr p.e.

⑥ V3000 ; dil 10^{-2}

VIII

4	8	12	18
22	26	44	80 hr p.e.

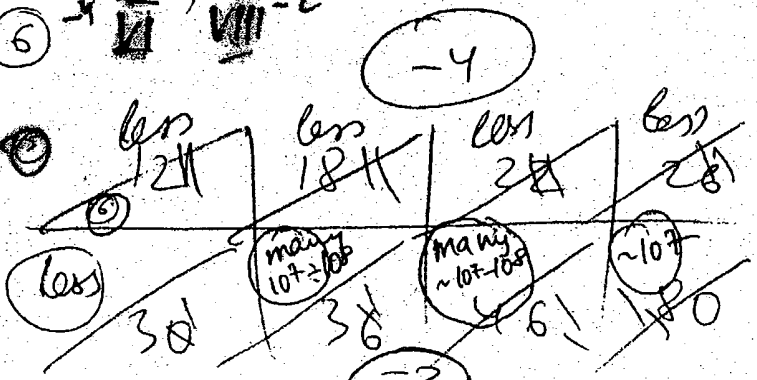
Difficult to count since VEE often when adjacent

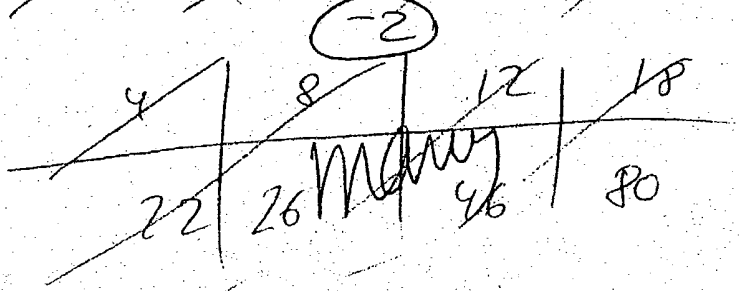
⑤ $^{-4}$ V , VII $^{-2}$

⑥ $^{-4}$ VI , VIII $^{-2}$

10/8/94 Saturday

1.





4 sides

LSN ~~10~~ 10^{-1} (NC)

IX

12	18	22	26
practically nothing			

65

10/9/94

INFECTION with supernatants from electroporation 10/6

IFAS

a-Lassa

	8 hrs p.e.	26	34	44
① LSN+Pl-5; dil 10^{-3}	0	4	5	$2 \cdot 10^4$/ml
② LSN+RSR; dil 10^{-3}	0	5	7	$14 \cdot 10^4$/ml

	8	26	34	44
③ Pl-5; dil 10^{-1}	0	0	0	0
④ V3000+LSN; dil 10^{-4}	0	0	$3 \cdot 10^5$	$6 \cdot 10^5$/ml

a-VEE

	8	26	34	44
① LSN+Pl-5; dil 10^{-3}	0	0	0	0
② LSN+Pl-5; dil 10^{-1}	0	0	0	0

	p	26	34	44
③ Pl-5; dil 10^{-1}	0	0	0	0
④ V3000+LSN; dil 10^{-4}	0	at	~23	~$23 \cdot 10^5$ foci/ml

10/11/94
Tuesday

Plaque assay

Samples

1	10/6; 8 hrs p.e.	LSN+V3000	10^{-4} dilution	0
2	-"- 26	-"-	-"-	0
3	-"- 34	-"-	-"-	0
4	-"- 44	-"-	-"-	0
5	10/2; 8 hrs p.e.	LSN+V3000	10^{-4} dilution	0
6	-"- 26	-"-	-"-	0
7	-"- 34	-"-	-"-	0
8	-"- 44	-"-	-"-	0
9	-"- 80	-"-	-"-	0
10	10/2; 8 hrs p.e.	V3000	10^{-4} dilution	0
11	-"- 26	-"-	-"-	2 + 0 plaques
12	-"- 34	-"-	-"-	1 + 0 plaques
13	-"- 44	-"-	-"-	1 + 0 plaques
14	-"- 80	-"-	-"-	0
			-"-	0

66

TRANSCRIPTIONS

OK

	LSN	V300W	P1-5	P2-1
water	8.8	8.8	3.9	5.4
5X Buf	8	8	8	8
25mM NTP-G	12	12	12	12
25mM cap	4.8	4.8	8.4	4.8
20x A/NotR	0.7	0.7	2	4
43 U/µl T7 (1:1 ethanol/water)	1	1	1	1
100 mM GDP	0.7	0.8	0.7	0.8
10x Enzymix	4	4	4	4
	40	40	40	40

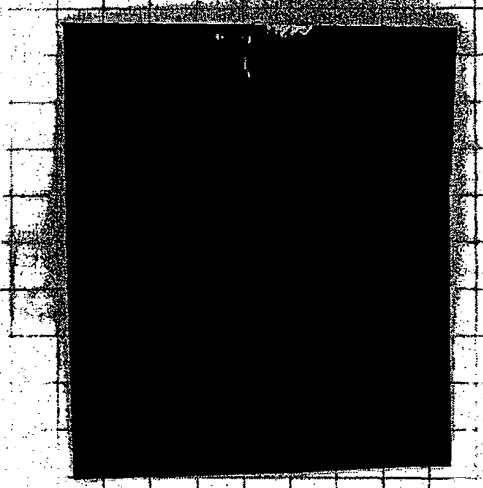
94
ersday

} LSN 0.2%

} V300W 0.2%

} P1-5 0.3%

} P2-1 0.2%

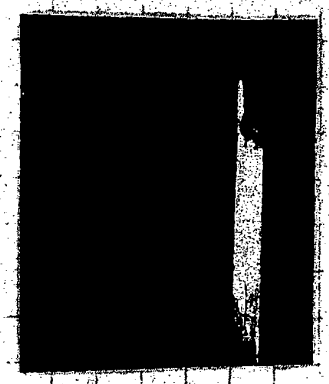


6

TRANSCRIPTIONS

	4 S N	~~V3000~~ OK	P1-5 ~ok
water	8.8	8.7	4.7
5X Buf	8	8	8
25 mM MP/G	12	12	12
25mM cap	4.8	4.8	8.4
20nA/Not-I	1	1	1.5
4 3 CTP	0.7	0.7	0.7
100 mM GTP	0.7	0.8	0.7
10 x Enzymix	4	4	4
	40	40	40

4 hrs 37°C



lassa 0.4%

V3000 4.2%

P1-5 6.8%

1µl of L8N/NotI

68

10/13/1994

TRANSCRIPTIONS

LSN OK

H2O	9.4
5× Buf	8
25 mM NTP/-G	12
25 mM cap	4.8
20 U RNasin	0.4 0.5
U3 GTP	0.7
100 mM GTP	0.7
10× Enzymix	4

6.2%

[redacted]

TRANSFECTIONS

	LSN (µl)				V3000 (µl)			P1-5 (µl)	P2-1 (µl)
	44	1.3	1.5	6.2	0.9	4.2	2.2	6.8	3.2
① LSN + V3000	5	10	–	6	5	12	5	–	–
② LSN + P1-5	5	10	–	6	–	–	–	10	–
③ LSN + P2-1	5	10	–	6	–	–	–	–	23
④ LSN + V3000	3	3	10	6	5	12	5	–	–
⑤ LSN + V3000	3	3	10	6	5	12	5	–	–
⑥ P1-5	–	–	–	–	–	–	–	10	–

0.4990

10/14/1994

Supernatants are taken

10/13	11:~~30~~ pm	(5 hrs p.e.)	cells are attached many cells on all of them alive
10/14	8:30 am	(14 hrs p.e.)	all cells are attached, it seems, that for many cells concentration may be high, some areas of clumped cells
	14:30 pm	(20 hrs p.e.)	
	do: 30 pm	(26 hr p.e.)	
10/15	8:30 am	(38 hr p.e.)	begin to "swim" ①-⑤ → all terminated collected

IFA for evaluation of transfection efficiency
(14 hrs p.e.) → LSN and V3000
in wells w/ samples ①, ② & ③ (LSN + Helpers)
cells monolayer sprinked, some of the slide ⇒ the cells are washed or not attached and were removed when slides were put into washing trays w/ PBS
too many cells (?!)

a-lassa

①	②	③	
~80%	1 hr cells	3 hr cells	/
~80% ④ ⑤	"0" ⑥	/	/

a-VEE

① 1 hr cells	② 2 hr cells	③ 3 hr cells	/
70-80% ④ ⑤	"0" ⑥	/	/

TRANSCRIPTIONS

	LSN	V3000	P2-1	RSR	P1-
water	18.8	8.7	5.4	3.9	4.1
5x buf	16	8	8	8	8
25mM NTP/-G	24	12	12	12	12
25mM cap	9.6	4.8	4.8	8/4	8/4
DNA/NotI	1	1	4	2	1.5
43 U/µl	1.3	0.7	1	0.9	0.7

10/14 8:30 am (14 hrs p.e.) all cells are attached / it seems, there too many cells → concentration may be high, some areas of eliminated cells

14:30 pm (20 hrs p.e.)

20:30 pm (26 hrs p.e.) left them to "swim" ①÷⑤

10/15 8:30 am (38 hrs p.e.) → all supernatants collected

A 090

IFA for evaluation of transfection efficiency (14 hrs p.e.) → LSN and V3000

in wells w/ samples 1, 2 & 3 (LSN + Helpers)

cell monolayer shrinked, some of the cells do → the are washed as not attached and were removed when slides were put into washing tray w/ PBS

too many cells (??)

a-lassa

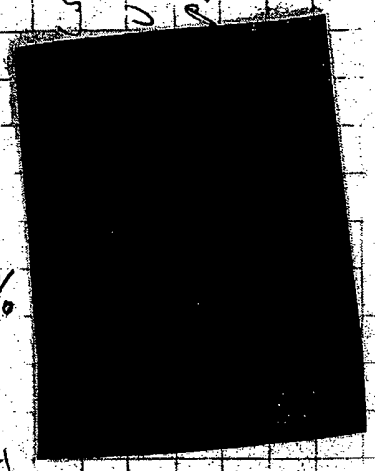
① ~80%	② two cells	③ two cells	/
~80% ④ ⑤	⑦ ⑥	/	/

a-VEE

① two cells	② two cells	③ two cells	/
~80% ④ ⑤	⑦ ⑥	/	/

TRANSCRIPTIONS

	LSN	V3000	P2-1	RSR	PI-
water	18.8	9.7	5.4	3.9	4.1
5x buf	16	8	8	8	8
25mM NTP/G	24	12	12	12	12
25mM cap	9.6	4.8	4.8	8/4	8/4
DNA/NotI	1	1	4	2	1.5
H3 WP	1.3	0.7	1	0.9	0.7
100mM GTP	1.4	0.8	0.8	0.7	0.7
10 x Enzyme	8	4	4	4	4



10/15/1994

Infection w/ supernatants of BHK cells
1 hr at 37°C infection in 100 µl of solution
(various dilutions), + 300 µl of medium,
incubate at 37°C

1 ml medium added (500 µl)

9/16/94

		③CSN+P1-5 5 hrs p.e. 10⁻⁴ dilution	14 hrs	22 hrs	26 hrs
I PFAs	I	wt	wt	"0"	2·10 (±25%) i.u./ml

a-Case

38 hrs p.e	⑥ 5 hr coda.	① 6 hr 10⁻² dil (many)	nc cells, no supernatants, last medium
2·10⁵ (±25%)	"0"		"0"

		②CSN+P1-5 5 hrs 10⁻⁴ dil	14 hr	22 hr	26 hr
II		"0"	"0"	1·10 (±25%)	6·10⁵ (±25%) i.u./ml

38 hr	nc	38 hr 10⁻²	nc
6·10⁵ (±25%)	"0"	(many)	"0"

			10⁻⁴ dil	14 hr	22 hr	26
III	③ CSN+P2-1	all negative	5 hr "0"	0	0	hr 0

38 hr	nc	38 10⁻² dil	5 hr 10⁻² dil
0	0	0	0

IV

④+⑤	8hr	14hr	20hr	26hr
10^{-4} dil	0	0	0	0

38hr	⑥ - 26hr conc.	④⑤ - 38hr 10^{-2} dil.
0	0	$1.3 \cdot 10^{6}$ c.u./ml

a-VEE

① - 14hr 10^{-4} dil.	20hr	26hr	38hr
0	1.10^{5}	1.10^{6}	$5 \cdot 10^{6}$

② - 14hr	20	26	38hr
10^{-2} dil	0	0	0

VI

③	14hr	20	26	38hr p.e.
10^{-2} dil	0	0	0	0

④⑤ - 38hr	① - 5	① - 5	⑥ - 38hr conc.
10^{-4}	10^{-4}	10^{-2}	
1	0	0	0

TRANSFECTIONS 20^{00} 10/16/94 $1 \div 2 \cdot 10^{7}$ cells/ml resuspended 0.8 ml of cells, 2 x 0.8 kV, 10 min. RT, diluted to 15 ml w/ medium → 2 x T25 flasks

① LSN + V3000 LSN 6.2% 2.2 µl 1.3 V3000 2.86% 10 µl
1.5% 10.0 1.5 2.2% 15 µl
3.5% 10.00 3.5 6.16 µg
6.3 µg

② LSN + P1-5 P1-5 6.5% 9.5 µl (?)
6.46 µg

③ LSN + D4-1 D4-1 7.5% 8.4 µl (?)
6.3 µg

7:

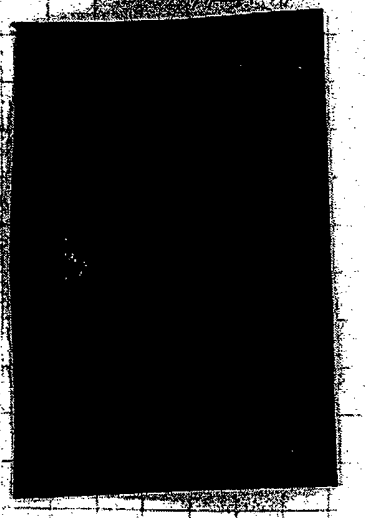
10/17/1994 IFA a-Lassa 13 hrs p.e.

① ~80% ② ~80%
③ ~80%

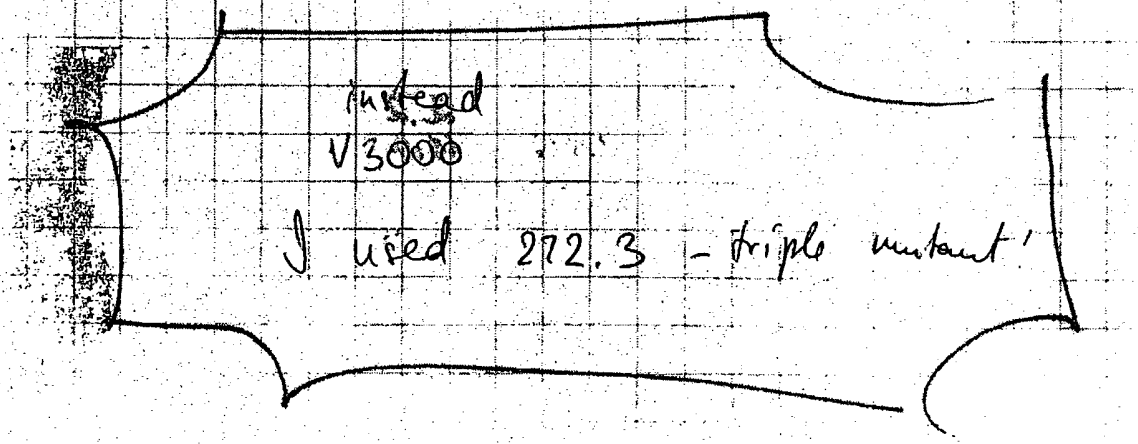
a-VEE

① ~80% ② ~1%
③ ~0%

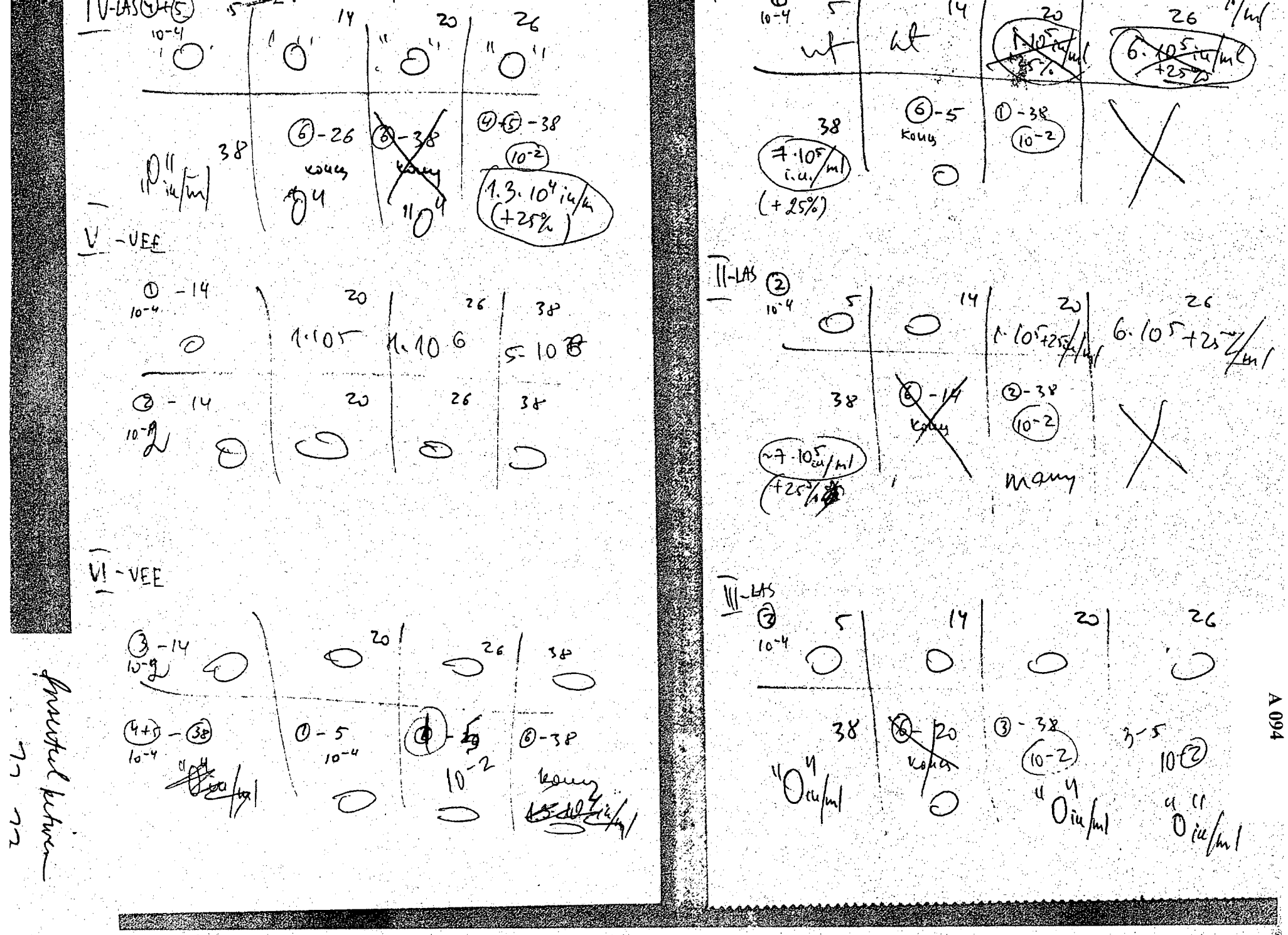
– why !!! monoclonal human (15 weeks p.e.) FA 21



denaturing RNA gel 1) P1-5 1 µl
2) P4-1 1 µl
3) RNA ladder 1 µl



10/18/94 Infection 22:00


IV-LAS
5
10-4
5
14
20
26
38
6-26
конц
6-38
конц
4+5-38
10-2
1.3·10⁴ i.u./ml
(+25%)
V - VEE
1-14
10-4
20
1·10⁵
26
38
2-14
10-2
20
26
38
VI - VEE
3-14
10-2
20
26
38
4+5-38
10-4
1-5
10-4
10-2
6-38
конц
II-LAS
2
10-4
5
14
20
26
38
6-14
конц
2-38
10-2
many
III-LAS
3
10-4
5
14
20
26
38
6-20
конц
3-38
10-2
3-5
10-2

A 094

73

10/19/1994

IFA of infection 10/18 - a-lassa (~15 hrs p.i.)

I LSN 238.3	12	24	36	48
	0	0	0.2·10⁵	2.6·10⁵

II LSN P1-5	12	24	36	48
	0	0	0	0

III LSN P4-1	12	24	36	48
	0	0	0	0

	12	24	36	48
	0	0	0	0

10^{-3} dilutions

~~Infection 10:30~~

10/20/1994

Large-scale plasmid DNA 3014 (~ 0.2 µg/µl)

3014 DNA cleavage 1) w/ Not I + BSA + ③ React
2) w/ Rsr II + Apa I + ⑤ React + BSA

Not I cleavage

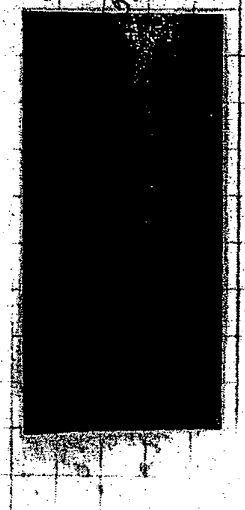
3014 DNA	200 µl
React ③ 10×	80 µl
50 mg/ml BSA	1 µl
water	520 µl
Not I	3 µl
	800 µl

at 37°C

Rsr II + Apa I cleavage

3014 DNA	200 µl
React ⑤ 10×	80 µl
50 mg/ml BSA	1 µl
water	520 µl
Apa I	
Rsr 2	
	800 µl

at RT



10/23/1994 1) Plaque assay

1	P1-5	$10^{-2} \div 10^{-4}$	14hr O	19.
2.			20hr O	15.
3.			26hr O	16.
4.			38hr O	17.
5	LSN+ 272.3	$10^{-5} \div 10^{-7}$	5hr O	18
6			14hr O	19.
~0.5.10⁵ (1kb)			20hr	20.
2·10⁵ (4kb) 8			26 hr	
~0.5.10⁵ (10kb)			38h	
10	LSN+ P1-5	$10^{-5} \div 10^{-7}$	5hrO	
11			14hrO	
12			20hrO	
13			26 hrO	

38hr	O	
LSN+ 272.3 $10^{-5} \div 10^{-7}$	O	
36 hr	$0.6-2 \cdot 10^6$ (2+2)	(6+
	$2-3 \cdot 10^6$ (2+2)	(29+
	$2-4 \cdot 10^6$ (3 and 2)	(5
60 hr	$4 \cdot 10^6$ (2 and 7)	(10+
85 hr	$2 \cdot 10^6$ (3+1)	(nt)

plaque titration 10/16/1994

Re: titration 10/16/94

1 — T4 — e.p. 5.9.

2) BHK-22 cells trypsinized, 6 tissue culture chambers covered at 10^5 cells per chamber. The rest of the T150 flask cells transferred into new T150 flask (not enough cells for splitting?)

3) DNA 1% TBE agarose 1) 3014/NotI/transcription grade 1 out of 70 µl
2) 3014/RsrII + ApaI/cleavage 5 µl
3) 1 kB MARKER

10/24/1994

1) 1% TBE Agarose 1) 3014/NotI/transcription grade 1 µl out of 140 µl
2) 3014/RsrII + ApaI 5 µl
3) KB marker

2) 3014/RsrII + ApaI: 2× phenol-chloroform, precipitate, wash 3× 75% EtOH, dry, dissolve in 90 µl water
10 µl 10× T4 pol
3 µl mix/NTP (30 mM total)
2 µl T4 polymerase 5 u/µl
RT 20 min. 2× Phenol chloroform, precipt; 3× 75% EtOH, dissolve

~ 0.5-10^5 (1 ml)
use undiluted!

10	GSN + PI-3	$10^{-5} \div 10^{-7}$	38 h
11			5 h 0
12			14 h 0
13			20 h 0
			26 h 0

A 097

2) BHK-22; cells trypsinized, 6 Lab-Tek slides covered at 10^5 cells per chamber. The rest of the 150 ml [illegible] transferred into new T80 flask (last one with many cells for specificity?)

3) DNA 1% TBE agarose 1) 3014/Not I/transcription grade 1 out of 70 µl
2) 3014/Rsr II + Apa I/ cleavage 5 µl
3) 1 kB MARKER

10/24/1994

1) 1% TBE Agarose 1) 3014/Not I/transcription grade 1 µl out of 140 µl
2) 3014/Rsr II + Apa I 5 µl
3) KB marker

2) 3014/Rsr II + Apa I; 2x phenol-chloroform, precipitate, wash 3x 75% EtOH, dry, dissolve in 90 µl water
10 µl 10x T4 pol
3 µl rNTP (30 mM total)
2 µl T4 polymerase 5 u/µl
RT 20 min. 2x phenol chloroform, precipit; 3x 75% EtOH, dissol
1 µl + 1% Agarose/TBE

P5: 2 µl + 14 µl water + 2 µl 6 mf + 2 µl T4 DNA Lig. 5 u/µl B/n +4°C

3) Electroporation BHK-23 (grown o/n after splitting)

(1.) V3000 = 272.3 about 7µg in 30µl — 20µl of 2.86% = 5.72 µg; 10 µl of 17% = 1.7 µg; 5 µl of 22% = 1.1 µg; 35µl — was zapped three times, 5 times finishing time constant 6.0

(2) LSN + P2-1
LSN 8.6µg in 25µl
P2-1 9µg in 25µl

(3) V3000 = 272.3 — 1.217 µg in 5µl

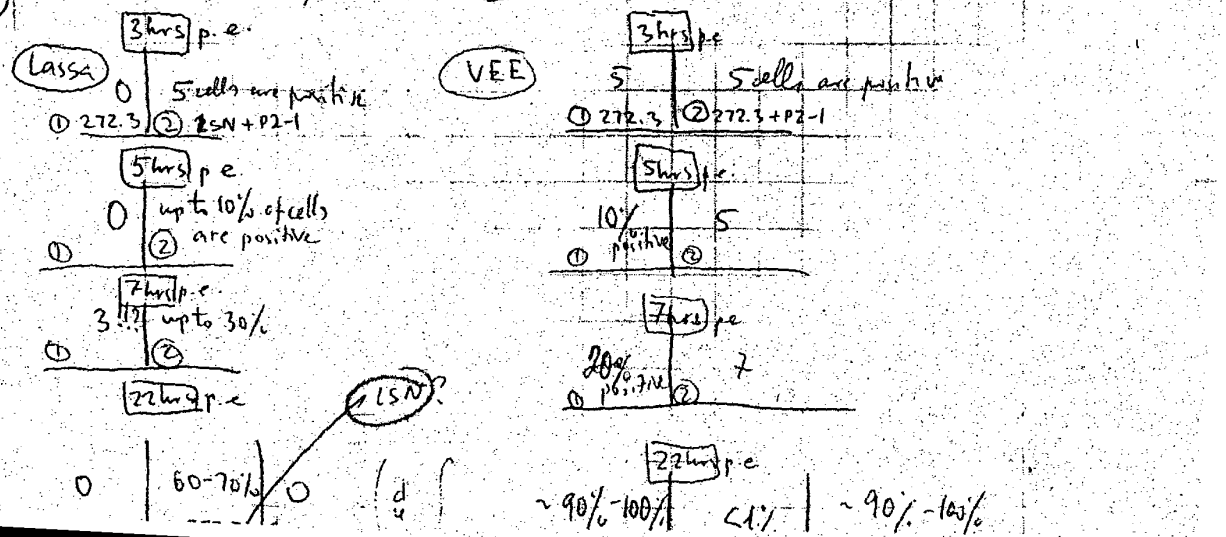
[redacted]

zapped cells have been transferred into 23 ml of medium, aliquots (400 µl) were taken for IFA, cells w/medium were transferred into 75 cm² flasks, incubation at 37°C 6% CO_2 started at 12:00

a. Sample for IFA is taken at 15.00 (3 hrs p.e.)
b. Sample for IFA is taken at 17:00 (5 hrs p.e.)
a. Sample for infectivity analysis is taken at 17:00 (5 hrs p.e.)
c. Sample for IFA is taken at 19:00 (7 hrs p.e.)
b. Sample for i.a. is taken at 19:00 (7 hrs p.e.)
d. 22hrs etc

10/25/1994

1) Plaque assay - counting ~~see~~ results in 10/23/94
2) IFA for LSN Ag and VEE



Vero - ... 1 J.

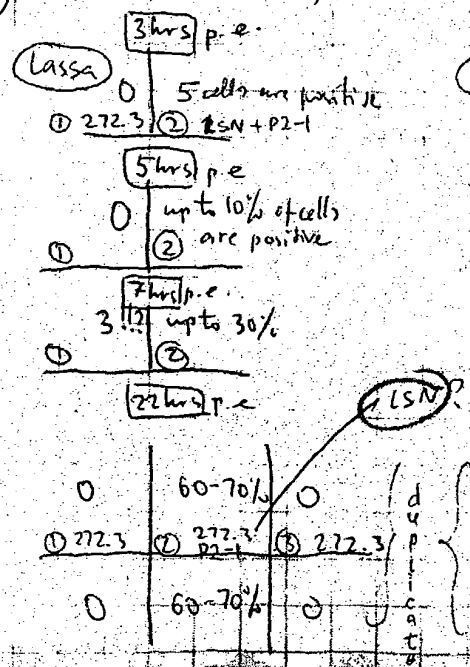
Lapped cells have been transferred into 23 ml of medium, aliquots (400 µl) were taken for IFA, cells w/medium were transferred into 75 cm² flasks, incubation at 37°C 6% CO_2 started at 12:00

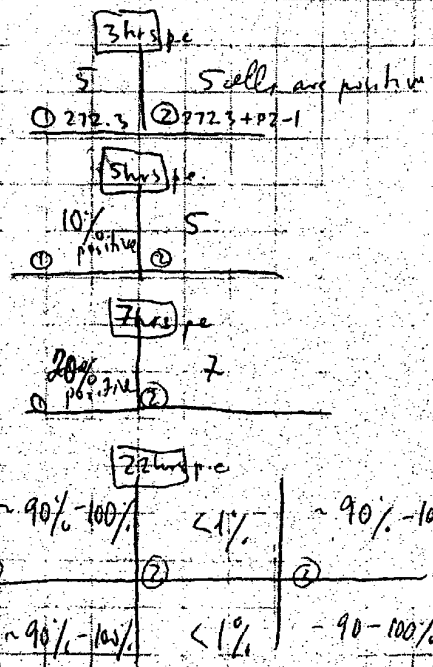
A 099

a. Sample for IFA is taken at 15:00 (3 hrs p.e.)
b. Sample for IFA is taken at 17:00 (5 hrs p.e.)
a. Sample for infectivity assay is taken at 17:00 (5 hrs p.c.)
c. Sample for IFA is taken at 19:00 (7 hrs p.e.)
b. Sample for i.a. is taken at 19:00 (7 hrs p.c.)
d. 22 hrs. etc

10/25/1

1) Plaque assay - counting ; see results in 10/23/99
2) IFA for LSV Ag and VEE





3) E. coli transformation P5

4) Infection of B cell cells

0/26/1994 1) ① 1) 272.3 + LSN 5hr p.e. undiluted sup. — Lassa Ag positive

2)	— " —	14 hrs	10^{-2}	0	(+10%) total 0
3)	— " —	20	10^{-2}	0	(+50%) 0
4)	— " —	20	10^{-3}	10	(+25%) $1.3 \cdot 10^{}$
5)	— " —	26	10^{-3}	0	(+40%) 0
6)	— " —	38	10^{-3}	0	(+75%) 0
7)	— " —	38	10^{-4}	7	(+80%) $1.2 \cdot 10^{5}$
8)	— " —	14	undil.	2	(+90%) $4 \cdot 10^{5}$
				26	(+30%) 34 cv./

② 1) P1-5 + LSN 5hr p.e. undiluted sup. — Lassa Ag positive

2)	— " —	14	10^{-2}	0	(+50%) total 0
3)	— " —	20	10^{-2}	2	(+90%) $4 \cdot 10^{}$
4)	— " —	20	10^{-3}	20	(+90%) $4 \cdot 10^{4}$
5)	— " —	26	10^{-3}	2	(+40%) $3 \cdot 10^{4}$
6)	— " —	38	10^{-3}	7	(+50%) $1.4 \cdot 10^{}$
7)	— " —	38	10^{-4}	2	(+25%) $3.3 \cdot 10^{}$
8)	— " —	14	undiluted sup.	10	(+90%) $2 \cdot 10^{6}$
				more	(+20%)

14) undiluted sup. P1-5 + LSN 5; 20; 26; and 38 hrs p.e. no VLP positive cells

③

1)	272.3 + LSN	12 hrs p.e.	10^{-1} undiluted sup	0	(+50%) 0
2)	"	24	10^{-1} (10^{-2})	100	(+50%) $2 \cdot 10^{}$
3)	"	24	10^{-3}	0	(+60%) 0
4)	"	36	10^{-3}	2	(+50%) $4 \cdot 10^{}$
5)	"	48	10^{-3}	11	(+40%) $2 \cdot 10^{}$
6)	"	60	10^{-3}	13	(+80%) $2 \cdot 10^{}$
7)	"	85	10^{-3}	24	(+70%) $4 \cdot 10^{}$
8)	"	12	undiluted	10	(+50%) 20
					150

2) (P5) - many clones ; inoculated 16 2ml-cultures
(P5k) - few clones

3) Cleavage 3014 DNA Kpn I

5) — " — 26 — 10⁻³ — 0 — (+75%) — 0
6) — " — 38 — 10⁻³ — 7 — (+80%) — 1.2
7) — " — 38 — 10⁻⁴ — 2 — (+90%) — 4
8) — " — 14 — undil. — 26 — (+30%) — 3

Cassa Ag positive

② 1) P1-5+CSN 5hr p.e — undiluted sup. — 0 — (+50%) — 4
2) — " — 14 — 10⁻² — 2 — (+90%) — 4
3) — " — 20 — 10⁻² — 20 — (+90%) — 4.1
4) — " — 20 — 10⁻³ — 2 — (+40%) — 3.1
5) — " — 26 — 10⁻³ — 4 — (+50%) — 1.4
6) — " — 38 — 10⁻³ — 2 — (+25%) — 3.3
7) — " — 38 — 10⁻⁴ — 10 — (+90%) — 2.1
8) — " — 14 — undiluted sup. — 0 — (+10%)

Cassa Ag positive cells

③ 1) 272.3+CSN 72hrs p.e — 10 undiluted sup — 0 — (+50%)
2) — " — 24 — 10⁻¹ (10⁻²) — 100 — (+50%) — 2.1
3) — " — 24 — 10⁻³ — 0 — (+60%)
4) — " — 36 — 10⁻³ — 2 — (+50%) — 4
5) — " — 48 — 10⁻³ — 11 — (+40%) — 2
6) — " — 60 — 10⁻³ — 13 — (+80%) — 2
7) — " — 85 — 10⁻³ — 24 — (+70%) — 4
8) — " — 12 — undiluted — 10 — (+50%) — 2

15

2) (P5) - many clones ; inoculated 16 2ml-cultures
(P5K) - few clones

3) Cleavage 3014 DNA Kpn I

50 µl — 10x ⑤ buffer
1 µl — 50 mg/ml BSA
20 µl — 3014 DNA
930 µl — H₂O
3 µl — Kpn I

10/16/94

a-lassa

undil.	0	10	0
+10% 1-5	+50% 1-14 10^{-2}	+25% 1-20 10^{-2}	+40% 1-20 10^{-3}
(1) 0			
+75% 1-26 10^{-3}	+80% 1-38 10^{-3}	+90% 1-38 10^{-4}	+30% 1-14 undil
0	7	2	26

(2) 0 2-5 undil	+90% 2-14 10^{-2}	+90% 2-20 10^{-2} 20	+10% 2-20 2 10^{-3}
+50% 2-26 10^{-3} (7)	+25% 2-38 10^{-3} (27)	+90% 2-38 10^{-4} 10	+10% 2-14 many undil

10/16

(1)	0 1-12 10^{-2}	+50% 1-24 100 10^{-1}	0 1-24 10^{-3}	2 1-30 10^{-3}
(3)	+40% 1-48 10^{-3} (14)	+80% 1-60 10^{-3} (13)	+70% 1-85 10^{-3} 24	+50% 1-12 (10) undil

(4)

a-VEE

undiluted			
(2) 0 2-5 50	2-20 50	2-20	+50%
2-28	2-38		

Found at pg 76-77

10/27/94

1) mini-preps of P5 1÷16 ; cleavage with Kpn I o/n
   Nos 1,2,8 – 1% gel (uncleaved)

2) 3014 Kpn I – 1% gel

(P6)

3014 Kpn I – 2 µl
10 x Buffer but 2 µl
H2O 14 µl
Ligase 2 µl 4°C - o/n

[redacted]

10/28/94

1) cleavage of P5-1; 2; and 3014 with Sma I
   (P5-1) is OK = Δ Xba I Kpn I of 3014

[redacted]

P5-1/P5-2/3014/KB

10/29/94

1) Maxi-prep isolation of DNA P5-1
   Cleavage with Not I
2) Transformation P6 (3014 Δ Kpn I) into Escli BH5α

10/30/94

1) inoculated 2-ml LB cultures P6 1÷16
2) P5-1/Not deproteinated (7x), precipitated (-70°C) o/n

10/31/94

1) P6 1÷16 cells are frozen, mini-preps, cleavage o/n at 37°C Xba I Kpn I
2) P5-1/Not precipitated, washed 3x 75% EtOH, dissolved in 40 µl water

1) 1% TBE Agarose gel : P6 1÷16 Xba I Kpn I ; P5-1/Not I

[redacted]

P5-1/

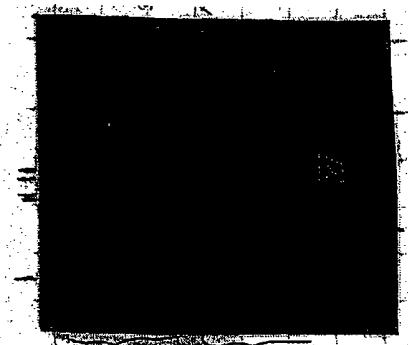
P6-1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 3014

[redacted]

OK

Cleavage (P6-2) : 1) Apa I BstE II
2) Apa I Kpn I

1% Agarose



2) TRANSCRIPTIONS 4 hrs at 37°C Apa/Kpn Apa/BstE

	LSN OK	3014 OK	P5-1 OK
water	18.8	8.7	4.7
5x BUF	16	8	8
25mM NTP/G	24	12	12
25mM cap	9.6	4.8	8.4
DRA/NOT I	1	1	1.5
H3 UTP	1.3	0.7	0.7
100 mM GTP	1.4	0.8	0.7
10x Enzyme mix	8	4	4

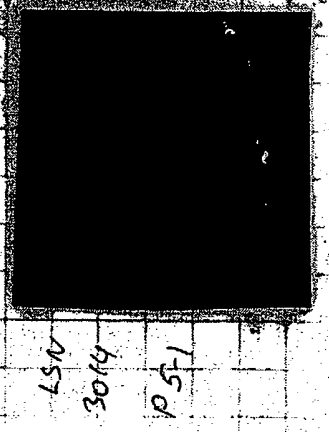
11/2/94

1) checking yesterday's transcription reactions

LSN 4.14%

P5-1 4.79%

3014 2.8%



2) TRANSCRIPTION 3014 as in 11/1/94

11/3/94 1) checking transcription 3014: 0.7%

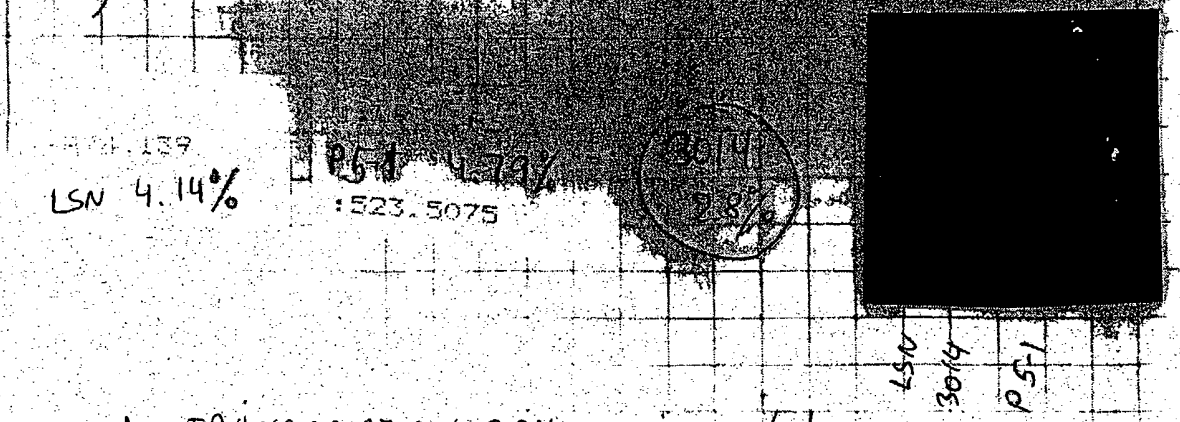
2) plaque assay

1	272.3/-5	10^{-2}÷-4	0	10	272.3/-27	10^{-4}÷-6	$6 \cdot 10^5$
2	LSN+P2-1/-5	10^{-2}÷-4	0	11	LSN+P2-1/-27	10^{-2}÷-4	0
* 3	272.3/-5	10^{-2}÷-4	0	* 12	272.3/-27	10^{-4}÷-6	$8 \cdot 10^6$
4	272.3/-7	10^{-2}÷-4	$2 \cdot 10^2$	13	272.3/-31	10^{-4}÷-6	$2 \cdot 10^5$
5	LSN+P2-1/-7	10^{-2}÷-4	0	14	LSN+P2-1/-31	10^{-2}÷-4	0

5x BUF	16	8	8
25mM MTP/G	24	12	12
25mM cap	9.6	4.8	8.4
2U RA/NOT-I	1		1.5
H3 UTP	1.3	0.7	0.7
100 mM GTP	1.4	0.8	0.7
10x Enzyme mix	8		

11/2/94

1) checking yesterday's transcription



LSN 4.14%

PSF1 4.79%

3014 2.8%

:523.5075

2) TRANSCRIPTION 3014 as in 11/1/94

11/3/94

1) checking transcription 3014: 0.7%

2) plaque assay

①	272.3/-5	$10^{-2}\div-4$	0	⑩	272.3/-27	$10^{-4}\div-6$	$6\cdot10^5$
2	LSN+P2-1/-5	$10^{-2}\div-4$	0	11	LSN+P2-1/-27	$10^{-2}\div-4$	0
*3	272.3/-5	$10^{-2}\div-4$	0	*12	272.3/-27	$10^{-4}\div-6$	$8\cdot10^5$
④	272.3/-7	$10^{-2}\div-4$	$2\cdot10^2$	⑬	272.3/-31	$10^{-4}\div-6$	$2\cdot10^6$
5	LSN+P2-1/-7	$10^{-2}\div-4$	0	14	LSN+P2-1/-31	$10^{-2}\div-4$	0
*6	272.3/-7	$10^{-2}\div-4$	$6\cdot10^2$	*15	272 3/-31	$10^{-4}\div-6$	$1.1\cdot10^7$
⑦	272.3/-22	$10^{-4}\div10^{-6}$	$2\cdot10^5$	⑯	272.3/-45	$10^{-4}\div-6$	$1.7\cdot10^6$
8	LSN+P2-1/-22	$10^{-2}\div-4$	0	17	LSN+P2-1/-45	$10^{-2}\div-4$	0
*9	272-3/-22	$10^{-4}\div10^{-6}$	$1.5\cdot10^6$				

*18	272.3/45	$10^{-4 \div -6}$	$1.2 \cdot 10^7$
(19)	272.3/-70	$10^{-4 \div -6}$	$4 \cdot 10^5$
20	LSV+P2-1/-70	$10^{-2 \div -4}$	0
*21	272.3/-70 hrs p.e.	$10^{-4 \div -6}$	$6 \cdot 10^6$

1) Maxi-Prep DNA isolation of P6-2 (2014 & KpnI) 18/4/94
Cleavage w/NotI: 800µl water, 100µl P6-2, 100µl 10x R③, 2µl 50 mg/ml BSA; 4µl 10U/µl NotI (BRL) 12⁰⁰÷

2) Plaque assay (cont'ed)

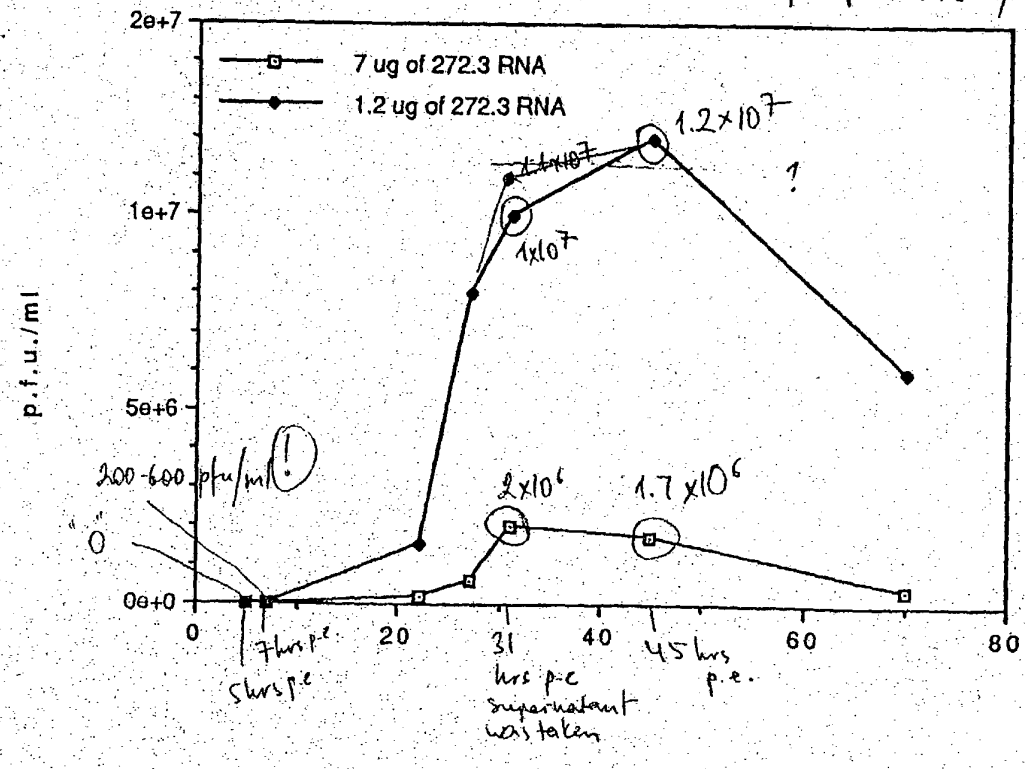
peter-11/3/94
LSV+P2-1
~~Cal/Dbo-4~~

	p.e., hours	272.3-7 ug	272.3-1.2 ug	
1	5.~~000~~	0.000	0.000	0.000
2	7.~~000~~	200.000	600.000	0.000
3	22.~~000~~	200000.000	1500000.000	0.000
4	27.~~000~~	600000.000	8000000.000	0.000
5	31.~~000~~	2000000.000	12000000.000	0.000
6	45.~~000~~	1700000.000	12000000.000	0.000
7	70.~~000~~	400000.000	6000000.000	0.000

Cells after were (-) for VEE Ag after e.p.; P2-1-dead?

BHK transfection with 272.3 RNA

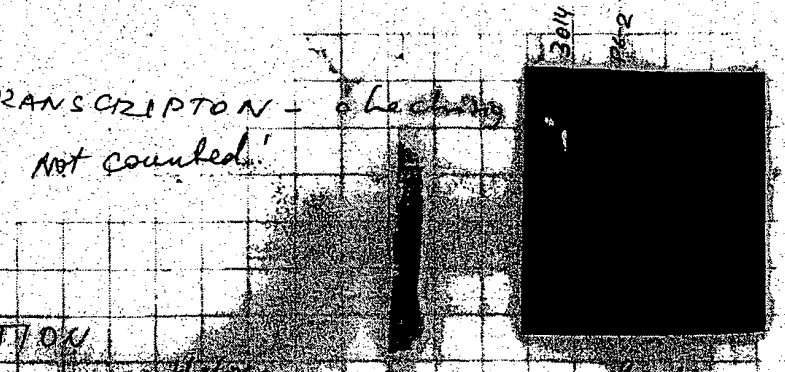
plaque assay



10/5/1994 TRANSCRIPTION

	3014/NotI	P6-2/NotI
Water	8.7	8.8
5x buf	8	8
25mM NTP/-G	12	12
25mM cap	4.8	4.8
DNA	0.9	1
U3 UTP	0.7	0.7
100mM GTP	0.8	0.7
10x Enz mix	4	4

37°C 16:00 – 19:10



11/6/94 1) unsuccessful TRANSCRIPTION – checking
Not counted!

2) TRANSFECTION

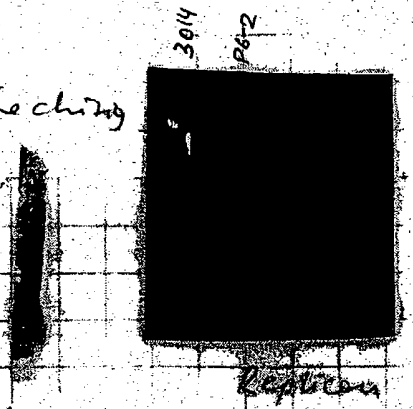
1)	3014	15/4.79 + 15/0.7 + 15/1	–
2)	3014 ∠	2µl/4.79 + 2µl/0.7 + 2µl/1	–
3)	CSN/3014	15/4.79 + 15/0.7 + 15/1	10/3.59 + 15/4.1
4)	CSN/P5-1	18/4.8	10/3.59 + 15/4.1
5)	P5-1	18/4.2	–
6)	(CSN) P6-2	18/1	10/3.59 + 15/4.1
7)	P6-2	18/1	–
8)	NC (just cells electroporated)	–	–

1130 incubation began

5x buf	8	8
25mM NTP/-G	12	12
25mM Cap	4.8	4.8
DNA	0.9	1
U3 URP	0.7	0.7
100 mM GTP	0.8	0.7
10x Enzmix	4	4

37°C 16:00 – 19:10

11/6/94 1) upstream TRANSCRIPTON - a few chains Not counted!



2) TRANSFECTION

		Helper	Reporter
1)	3014	15/4.79 + 15/0.7 + 15/?	—
2)	3014 ∠	2µl/4.79 + 2µl/0.7 + 2µl/?	—
3)	LSN/3014	15/4.79 + 15/0.7 + 15/?	10/3.59 + 15/4.1
4)	LSN/P5-1	18 ~~20~~/4.8	10/3.59 + 15/4.1
5)	P5-1	18 ~~20~~/4.8	—
6)	(SN) P6-2	18/?	10/3.59 + 15/4.1
7)	P6-2	18/?	—
8)	NC (just cells (Electroporated)	-	—

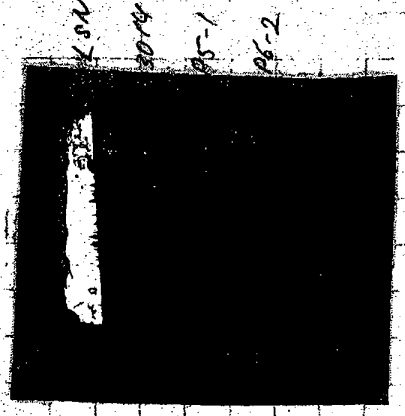
1130 incubation began

Cells did not attach to surface. Caused by infection? bacterial

11/7/94

TRANSCRIPTIONS

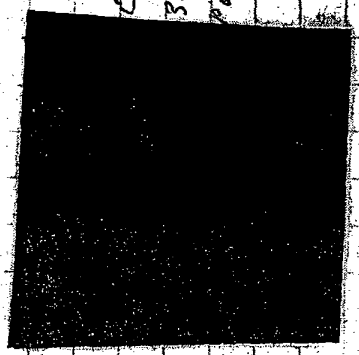
	LSN	3014	P5-1	P6-2
water	18.8	8.7	4.7	8.8
5x Buf	16	8	8	8
25mM NTP/-G	24	12	12	12
25mM cap	9.6	4.8	8.4	4.8
DNA/NotI	1	1 (0.9)	1.5	1
H3 UTP	1.3	0.7	0.7	0.7
100 mM GTP	1.4	0.8	0.7	0.7
10x Enzyme	8	4	4	4
				37°C 3.5hr
	7.7%	3.3%	4.8%	4.9%



11/8/94

TRANSCRIPTIONS

	3014	LSN	P6-2
water	8.7	8.8	8.8
5x Buf	8	8	8
25mM NTP/-G	12	12	12
25mM cap	4.8	4.8	4.8
DNA/Not	0.9	0.5	1
H3 UTP	0.7	0.7	0.7
100 mM GTP	0.8	0.7	0.7
10x Enz mix	4	4	4
	2.4%	5.9%	5.7%



8)

11/9/94 Checking BHK cells

11/8/94 cells were ~ healthy, ~ 50% confluent, OK

11/9/94 cells contain granules, overgrown, many dead cells in medium, 70% confl. (Mike) not confluent (Randy), more than confluent (George) Trypsin: Zahn's Cow, suspended with difficulties

1 ml of trypsinized cells in stop solution (medium) + 1 ml of Trypan Blue,

Count cells in 'hemocytometer',

2 out of 64 are dead (blue),

overall amount of cells $2 \cdot 10^7$ per +150 flask

Cells prepared as usually for e-poration

0.8 ml of cells e-porated w/o RNA

0.8 ml of cells not e-porated

Seeded into 75 cm² flasks ~~11~~ 20 :

attached cells: 13 : 20 ~ 30% of surface

TRANSFECTIONS:

1) 3014 ~~11~~/8.4 + ~~11~~/3.3

2) 3014 1/8.4 + 1/3.3

3) 3014 + LSN 10/5.9 + 10/4.14

4) P5-1 + LSN 10/5.9 + 10/4.4

Seeded into 75 cm² flasks 15:00

Supernatant	IEA
1) 5 hr 30 min p.e.	1) 5 hrs p.e.
2) 7 hr 30 min p.e.	2) 7 hrs 30 min p.e.
3) 19 hr p.e.	3) 19 hrs p.e.
4) 27 hrs p.e.	
5) 41 hrs p.e.	
6) 96 hrs p.e.	

IFA:

11/10/94

a-Lassa conjugate: 5 hrs p.e. 1) sample 0 positive
2) 0 positive
3) few positive
4) 0–1% of cells are positive (many dead)

7 hrs p.e. 1) 0
2) 0
3) ~3% positive
4) ~5% positive

19 hrs p.e. 1) 0
4) 0
3) ~5% positive
4) ~90% positive !!!

a-VEE 5 hrs p.e. 1) 50% positive
2) 10%
3) ~20%
4) ** ~1%

7 hrs p.e. 1) 90%
2) 50%
3) 90%
4) ** ~5%

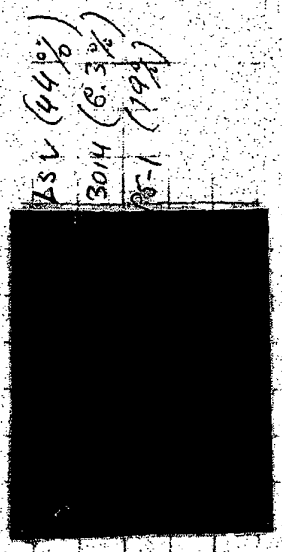
19 hrs p.e. 1) 100%
2) 100%
3) 100%
4) ** ~20% !!!

Resume: synthesis of Lassa Ag starts in about 5 hrs in these cells (in earlier experiment, in about 3 hr: does it depend on helper virus or quality of cells?)
synthesis of VEE Ag starts earlier than in 5 hrs p.e. (up to 50% of cells synthesize VEE Ag in 5.30 p.e.)
In 7 hrs p.e. expression of LSN Ag was similar in (LSN + 3014) and (LSN + P5-1) cells; but in 19 hrs p.e. there are 5% positive for LSN-Ag in (LSN + 3014) and

81

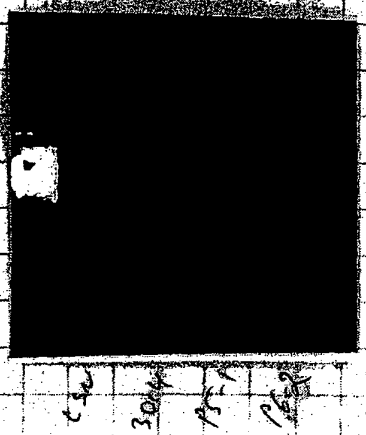
TRANSCRIPTIONS

	LSN	3014	P5-1
water	8.8	8.7	4.9
5x buf	8	8	8
25mM NTP/-G	12	12	12
25mM cap	4.8	4.8	8.4
RNA/Not	0.5	0.9	1.5
H3 ATP	0.7	0.7	0.7
100mM GTP	0.7	0.8	0.7
RX Buf	4	4	4



? LSN 0.5 µg/µl
3014 0.1 µg/µl
P5-1 0.3 µg/µl

10/14/94 3014 LSN, P1-5, and P6-2 RNAs are combined from different transcription reactions, concentration of RNA determined by gel electrophoresis and scintillation counting

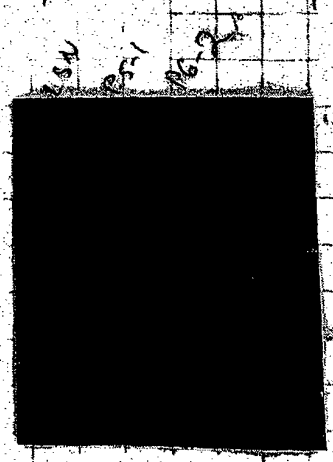


(0.3 ??) LSN ~ 0.8 µg/µl ~ 80 µl *
3014 ~ 0.2 µg/µl ~ 80 µl *
P5-1 ~ 0.6 µg/µl ~ 80 µl *
P6-2 ~ 0.1 µg/µl ~ 70 µl

NC O

TRANSCRIPTIONS

	LSN	P5-1	P6-2
waters	8.8	4.7	8.8
5× buf	8	8	8
25mM NTP/-G	12	12	12
25mM cap	4.8	8.4	4.8
DNA/Not	0.5	1.5	1
H3 ATP	0.7	0.7	0.7
100mM GTP	0.7	0.7	0.9
10× Buf	4	4	4



LSN 38% ?1

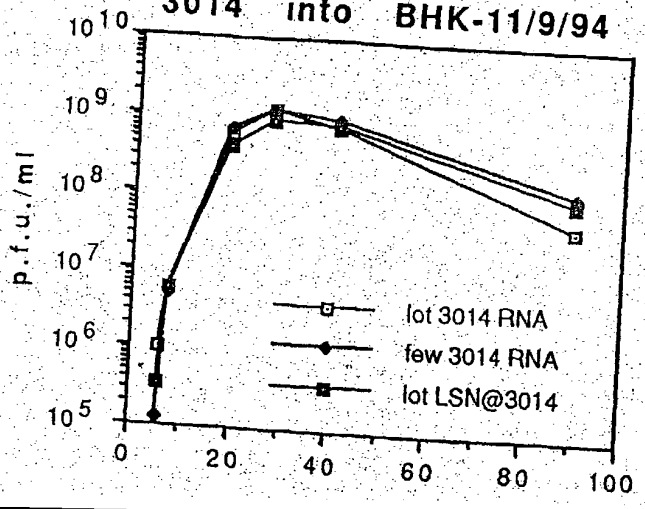
P5-1 35% ?1

P6-2 32% ?1

LSN	0.3 µg/µl	~ 35 µl
P5-1	0.3 µg/µl	~ 35 µl
P6-2	0.2 µg/µl	~ 35 µl

11/17/9

3014 into BHK-11/9/94



37h	1.2×10^{8}	(9)	undil	44		0
37h	$8.4\cdot10^{8}$	(14)	undil	90		0
41	7.3×10^{8}	3	NC	undil	23	0
41	9.2×10^{8}	4	NC	undil	31	0
41h	7.8×10^{8}	5	NC	undil	44	0
20	7.5×10^{7}	(26)	10/13	LSN P1-5	undil	5hr 0
20	1.3×10^{8}	27	undil			14hr 0
20	1×10^{8}	28	undil			20hr 0
5	0	29	undil			26hr 0

	8.8	4.7	5.8
	8	8	8
	12	12	12
	4.8	8.4	4.8
	0.5	1.5	1
	0.7	0.7	0.7
	0.7	0.7	0.9
	4	4	4

LSN 38% ?!

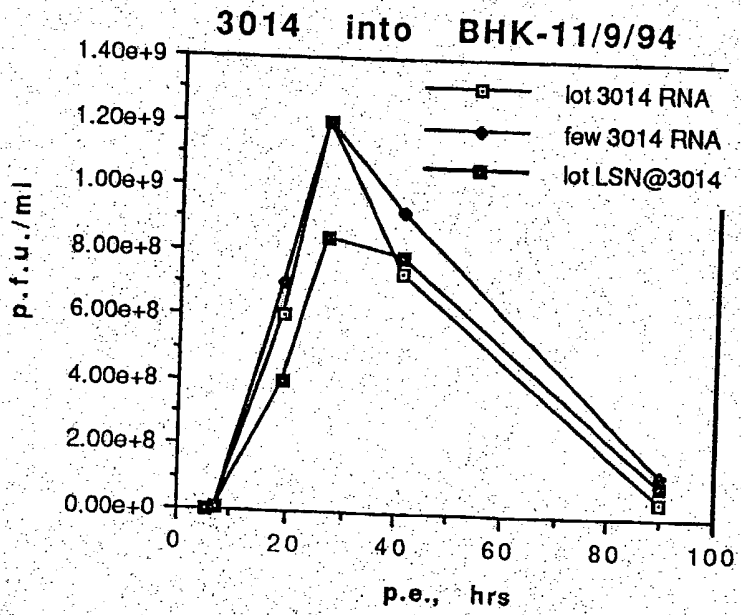
P5-1 35% ?!

P6-2 32% ?!

[redacted]

LSN	0.3 µg/µl	~35 µl
P5-1	0.[illegible] µg/µl	~35 µl
P6-2	0.2 µg/µl	~35 µl

11/17/



27 h	[illegible]	(25)	undil	44	0
27 h	4×10⁸	(24)	undil	90	0
41	[illegible]	NC	undil	23	0
41	9.2×10⁴	NC	undil	31	0
41 h	7.8×10⁵	NC	undil	44	0
90	4.5×10⁸	(26)	10/13 LSN P1-5 undil	5 hr	0
70	1.3×10⁸	(27)	undil	14 hr	0
70	1×10⁸	28	undil	20 hr	
50		29	undil	26 hr	0
10		30	undil	38 hr	0

26 ÷ 30 ep 10/13/94

1 ÷ 25 ep 11/9/94

H2O	8.8	4.7	5.8
5x buf	8	8	8
25 mM ATP/G	12	12	12
25 mM Cap	4.8	8.4	4.8
RNA Mix	0.5	1.5	1
RNase Inh	0.7	0.7	0.7
10 mM GTP	0.7	0.7	0.9
T7 x Pol	4	4	4

[redacted]

LSN 38% ?!

P5-1 35% ?!

P6-2 32% ?!

LSN	0.3 µg/µl	~35 µl
P5-1	0.[illegible] µg/µl	~35 µl
P6-2	0.2 µg/µl	~35 µl

11/12/94

Plaque assay

1 @ undil [illegible] $10^{-2} \div 10^{-4}$ [illegible]

28 hr	1.2×10^8	(9)	undil	44	0	
27	4.8×10^8	(4)	undil	90	0	
41	7.3×10^8	NC	undil	23	0	
41	4.2×10^8	NC	undil	31	0	
41	7.8×10^8	NC	undil	44	0	
90	4.5×10^8	(26)	10/13 LSN P1-5 undil	5 hr	0	
90	1.3×10^8	(27)	undil	14 hr	0	
90	1×10^8	28	undil	20 hr	0	
50		29	undil	26 hr	0	
10		30	undil	38 hr	0	

26 ÷ 30 e.p 10/13/94

1 ÷ 25 e.p 11/9/94

data 3014-11/9/94

	p.e., hrs	(1) lot 3014 RNA	(2) few 3014 RNA	(3) lot LSN@3014
1	5.500	1000000.000	130000.000	350000.000
2	7.500	6000000.000	5000000.000	6000000.000
3	19.000	00000000.000	00000000.000	00000000.000
4	27.000	00000000.000	00000000.000	40000000.000
5	41.000	30000000.000	20000000.000	80000000.000
6	90.000	45000000.000	30000000.000	00000000.000

81

1% Agarose : pV300 (0.5 µl) ; 1 Kb

Concn. ~ 1 µg/µl

11/14/94 TRANSFECTIONS CEF cells (seeded yesterday)

1. 3014 (30 µl) ~ 6 µg

2. 3014 (2 µl) ~ 0.2 µg + 28 µl H₂O

RNA from 11/14/94 (***)

3. CSN (3 µl) ~10 µg + 3014 (30 µl) ~ 6 µg

4. CSN (3 µl) ~10 µg + 3014 (1 µl) ~ 0.2 µg

5. CSN (3 µl) ~10 µg + P5-1 (3 µl)

6. CSN (3 µl) ~10 µg + P6-2 (17 µl) ~4 µg

7. P6-2 (17 µl) ~4 µg

8. NC / (no RNA) / 1/2 electr.

next time take 2×10^7 cells/ml

10^7 cells/ml

0.8 ml cells + RNA

seeded into 25 cm² flasks

CEF in 5 hrs p.e. seem to be adhered to plastic but many cells are rounded, as they are not very well attached.

25 cm² flask seems to be just OK for 10^7 cell/ml for CEF (better take 2×10^7 cells)

for IFA transfection effic.
1) 5 hrs p.e.
2) 14 hrs p.e.

for Plaque assay and i.u.
+1) 6 hrs p.e. (0 hr)
+2) 14 hrs p.e. (8 hr)
+3) 22 hrs p.e. (16 hr)
+4) 30 hrs p.e. (00 hr)
+5) 38 hrs p.e. (8 hr)
+6) 46 hrs p.e (16 hr)

11/17/94 TRANSFECTIONS CEF cells (seeded ~~yesterday~~)

1. 3014 (30μl) ~ 6μg

~~2.~~ 2. 3014 (2μl) ~ 0.2μg + 28μl H₂O

RNA from 11/14/94 (***)

3. LSN (3μl) ~10μg + 3014 (30μl) ~ 6μg

4. LSN (3μl) ~10μg + 3014 (1μl) ~0.2μg

5. LSN (3μl) ~10μg + P6-1 (3μl)

6. LSN (3μl) ~10μg + P6-2 (47μl) ~4μg

7. P6-2 (47μl) ~4μg

8. NC / (w RNA) 1/2 Kreton

next time have 2×10^7 cells/ml

10^7 cells/ml

0.8 ml cells + RNA

Seeded into ~~2~~ 25 cm² flasks

CEF in 5 hrs p.e. seem to be adhered to plastic but many cells are wounded, as they are not very well attached

25 cm² flask seems to be just OK for 10^7 cell/ml for CEF (better take 2×10^7 cell

for IFA transfection effic.
1) 5 hrs p.e.
2) 14 hrs p.e.

for Plaque assay and i.u.
+1) 6 hrs p.e. (0^hr)
+2) 14 hrs p.e. (8^hr)
+3) 22 hrs p.e (16^hr)
+4) 30 hrs p.e. (00^hr)
+5) 38 hrs p.e (8^hr)
+6) 46 hrs p.e (16^hr)
+7) 54 hrs p.e (00^hr)
+8) 70 hrs p.e (16^hr)

11/18/94

IFA

1) (15) hrs p.e

	a-Lassa	a-VEE
1. 3014(6μg)	"0"	20-30%
2 3014(0.2μg)	"0"	few
3 LSN + 3014(6μg)	few	20-30%
4. LSN + 3014(0.2μg)	few	few
5. LSN + P5-1	few	few
6 NC	"0"	"0"

2) (14) hrs p.e.

	a-Lassa	a-VEE
1. 3014 - 6μg	"0"	100%
2. 3014 - 0.2μg	"0"	90 ÷ 100%
3. LSN+3014(6μg)	30-40%	100%
4. LSN+3014(0.2μg)	30-40%	90% - 100%
5. LSN+P5-1	30-40%	~10-20%
6. NC	"0"	"0"

Generally, cells are poorly attached to plastic 5 hrs p.e., many dead cells. Cells look much better, better attached to plastic in 14 hrs p.e.

Probably, need to use more LSN RNA for transfection (and P5-1?): RNA amount (*) is too high, in reality probably 300 ng/μl LSN

Generally, synthesis of either LSN Ag or VEE Ag is in 5 hrs p.e. (few positive cells), may be a little bit slower than in BHK cells.

Improvements of CEE use: 1) use more LSN RNA (3 times more)
2) take more cells (2×10^7 cells/ml)
3) make IFA ~ 20 hrs p.e.

11/21/94 BHK-21 cells (Mixe) were trypsinized and seeded at 80.000/chamber of 8-chamber LabTek slides 10:30 am (for i.v. analysis)

at 16:30 they are still very poorly attached to plastic - rounded, clumped etc. (!?)

Clearage o/n at 37°C 3014 : Apa I + Avr II

3014 DNA	50
10× React	50
Avr II & Apa I	some
water	400

pV3000 (1) inoculated with LB-Amp (2 ml) o/n at 37°C shaking

11/22/94 To L. Johnston

DNA P5-1	100 µl
NH4 Ac	15 µl
Ethanol	300 µl

-20°C and to John Smith

I.V. analysis

LabTek slide (1)

27 hrs p.e. 30 hrs p.e.

11/9 (1)	11/9 (3)	11/9 (4)	11/17 (1)
conc	10^{-4}	10^{-4}	conc
0	43	35	0
11/17 (3)	11/17 (4)	11/17 (5)	nc
10^{-4}	10^{-4}	10^{-4}	
3	0	0	0

(43), taken with factor of dil and 100 µl p inoculum mean 4.3×10^6 i.

(2)

11/9 (1)	11/9 (3)	11/9 (4)	11/17 (1)
conc 0	10^{-5} 3	10^{-5} 4	conc nc
11/17 (3)	11/17 (4)	11/17 (5)	
10^{-5} 0	10^{-5} 0	10^{-5} 1	0

clumped etc. (-)

Cleavage o/n at 37°C 3014 : Apa I + Avr II

3014 DNA	50
10× ReACT	50
Avr II & Apa I	some
water	400

pV3000 (1) inoculated into LB-Amp (2 ml) o/n at 37°C shak

11/22/94

To K. Johnston:

DNA P5-1	100 µl
NH_4Ac	15 µl
Ethanol	300 µl

-20° and to John Smith

I.C. analysis

Load TEC slide (1)

27 hrs p.e. 30 hrs p.e.

11/9 (1)	11/9 (3)	11/9 (4)	11/17 (1)
conc	10^{-4}	10^{-4}	conc
0	43	35	0
11/17 (3)	11/17 (4)	11/17 (5)	
10^{-4}	10^{-4}	10^{-4}	nc
3	0	0	0

(2)

11/9 (1)	11/9 (3)	11/9 (4)	11/17 (1)
conc 0	10^{-5} 3	10^{-5} 4	concnt
11/17 (3)	11/17 (4)	11/17 (5)	
10^{-5} 0	10^{-5} 0	10^{-5} 1	nc 0

(43), taken wi
factor of
and 100 µl
infection
means
4.3×10^6

blue shows amount of num of positive (CS

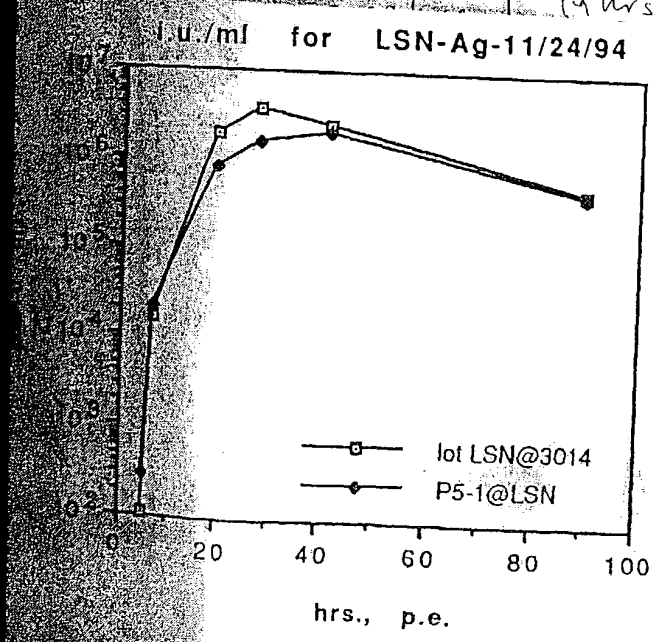
1 hr at 37°C infection w/ 100 µl of medium + supernatant at appropriate dilution + 300 µl of medium at 37°

15:00 - 8:00

1) heat treated (twice)
2) pre-incubated with 6 ul of supernatant

11/23/94

[redacted]



@3014-11/24 Thu, Nov 24, 1994 12:27 PM

11/25/94 Plaque assay (virus in Chick cells) from transfection 11/17

1	①	-6 hrs p.e.	5×10^5	11	②	-22	7×10^6	21	③ 38	1.5×10^7
2		-14 hrs	7×10^6	12		-30	1.1×10^7	22	-46	8×10^6
3		-22 hrs	8×10^6	13	3014	-38	1.3×10^7	23	-54	6×10^6
4		-30 hrs	1.5×10^7	14		-46	1.3×10^7	24	-70	3×10^6
5	3014	-38 hrs	1.8×10^7	15	0.2 μg	-54	1×10^7	25	④ -6	4×10^4
6	6 μg	-46 hrs	6×10^6	16		-70	7×10^5	26	-14	1×10^6
7	6 μg	-54 hrs	1.6×10^7	17	③	-6	3.8×10^5	27	-22	3×10^6
8		-70 hrs	3×10^6	18	LSN	-14	4×10^6	28	-30	5×10^6
9	②	-6	2×10^4	19	6 μg 3014 + 10 μg	-22	5×10^6	29	-38	4×10^6
10		14	5×10^6	20		-30	7×10^6	30	-46	6×10^6
								31	0.2 μg 3014 + 10 μg LSN	5×10^6
								32	-70	1.3×10^6

LSN + PS-1 { ⑤ -30 hrs 33 ; ⑥ -38 hrs 34 } "0"

NC { ⑥ -30 hrs

[redacted]

Clearance pV230. Bgal IR3+11 continued

Vero cells trypsinized & split into 4 150 flask and LabTek slides

11/26/94 ① TRANSFECTION CHIC 2×10⁷ cells/ml 14:00

i) LSN + PS-1 — 11/7 15μl → 15μl; marked 11/10, gel on 11/14 — plus 5μl from 10/4 (LSN, PS-1, P6-2)

2) LSN + P6-2 — 11/7 15μl → 25μl; marked, gel on 11/14

3) P6-2 (25μl) gel on 11/14

0.8ml + 10ml Comp. medium into 25 cm² flasks

Table **Analysis of the supernatants of BHK cells transfected with full length; and replicon and/or helper RNAs**

BHK cells

Helper	LSN Rep	i.u./ml, LSN	i.u./ml, VEE	p.f.u./ml
-	+	<100	n.t.	n.t.
272.3full length	-	n.t.	n.t.	1.2x10exp7
272.3full length	+	4-9x10exp5	n.t.	4x10exp6
272.3dRsrApa, P1-5	-	<100	<10	<100
272.3dRsrApa, P1-5	+	8-20x10exp5	<100	<100
272.3dKpn, P2-1	-	n.t.	n.t.	n.t.
272.3dKpn, P2-1*	+	n.t.	n.t.	n.t.
3014full length	-	<10	n.t.	1.2x10exp9
3014full length	+	4x10exp6	n.t.	8.4x10exp8
3014dRsrApa, P5-1	-	n.t.	n.t.	n.t.
3014dRsrApa, P5-1	+	4x10exp6	n.t.	<100
3014dKpn, P6-2	-			
3014dKpn, P6-2	+			

* does not function as helper, since no synthesis of VEE structural proteins was detected after electroporation with both RNAs, as determined by IFAs (for LSN Ag and VEE). Why it doesn't ????

CEF cells

Helper	LSN Rep	i.u./ml, LSN	i.u./ml, VEE	p.f.u./ml
3014full length	-	n.t.	n.t.	2x10exp7
3014full length	+	7x10exp4	n.t. confirmed →	2x10exp7
3014dRsrApa, P5-1	-	n.t.	n.t.	n.t.
3014dRsrApa, P5-1	+	2x10exp5	n.t.	<100
3014dKpn, P6-2	-			
3014dKpn, P6-2	+			

but at 10^{-3} and 10^{-4} dilution

5 hr p. infection

9

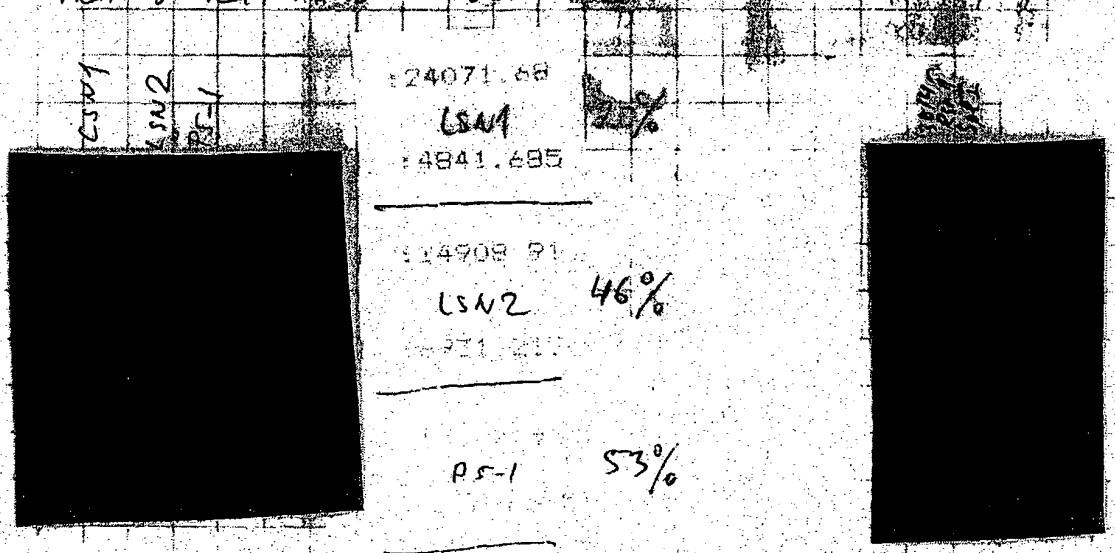
(3) DNA pV3000/ApaI+BsrGI 10 µl
Water 75 µl
10x T4 pol 10 µl
dNTP 3 µl
T4 pol. 2 µl

20 min RT
+ twice phenol-chlor precipitation

(4) DNA pV3000 10 | cDNA 3014 10
10x React 5 10 | 10x (5) 10
H2O 80 | H2O 80
KpnI 5 min 0/n at 37°C | BsrGI 2 µl/µg/37°C

1/28/94 Cleavage ok, phenolized 1x, precipitated

TRANSCRIPTION LSN and PS-1 as in 11/14



NC 0,6%

Ligations

P7 : pV3000 / RsrII/ApaI/T4 O

P8 : pV3000 / KpnI : many colonies

P9 : pV3000 / KpnI / T4 O

P10 : pV3014 / RsrII / SpeI / T4 : 10 colonies

DNA 2 µl
10 x Ligase buffer 2 µl
Mw 14 µl
Ligase 2 µl
O/n at 4°C

P7 (Rsr ApaT4
P8 (pV3000 KpnI
P9 (KpnI T4
P10 pV3014 Rsr SpeT4

- Ah !!!

! Double band(s) !

11/29/95

1) P10 phenolized, precipitated, cleaved w/ KpnI
aliquot – Dr 5 & CSh

2) TRANSFORMATION

3) RNA extraction from → 1) 3014 – ① – click Transformation 11/17
3x10⁶ pfu/µl → 2) P5-1 & CSN – ⑤
3x10⁵ cells/ml → 3) P1-5 & CSN
10⁶ cells/ml

RNA dissolved in 70 µl → -70°C

31/01/94 1) The rest of P7, P8, P9, P10 → dump flasks

2) a. CSN plasmid
b. 3026 - - - } 2ml LB-Ampl inoculated
c. P8 1÷14 inoculated and P10 1÷10 (clones) inoculated

3) TRANSFECTION

① CSN & P5-1 (30µl + 30µl)

② CSN & P5-1 (15µl + 15µl)

③ CSN & P5-1 (5µl + 5µl)

④ CSN & P6-2 (15µl + 15µl)

⑤ P5-1 (15µl)

⑥ P6-2 (10µl) - 75cm² flasks (20ml)

11²⁰ - 14²⁰ (27hrs p.e.)

1/02/94 IFA - a-Lassa and a-VEE (TRANSFECTION EFF.)
22 hrs p.e.

a-Lassa

①	②	③
~100%	~100%	~10%
④	⑤	⑥
~100%	0	0

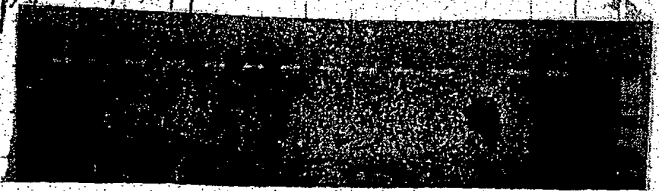
a-VEE

①	②	③
~100%	10%	100%
④	⑤	⑥
~70 ~80%	0	0

Mini-preps

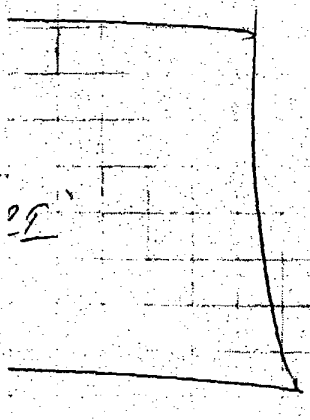
p10 1÷10

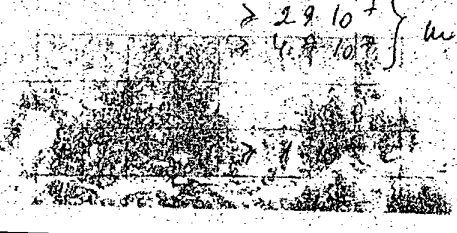
p8 1÷14



12/02/94

) or ? sent in November, 1994
by Nancy Davis & Bob Jones



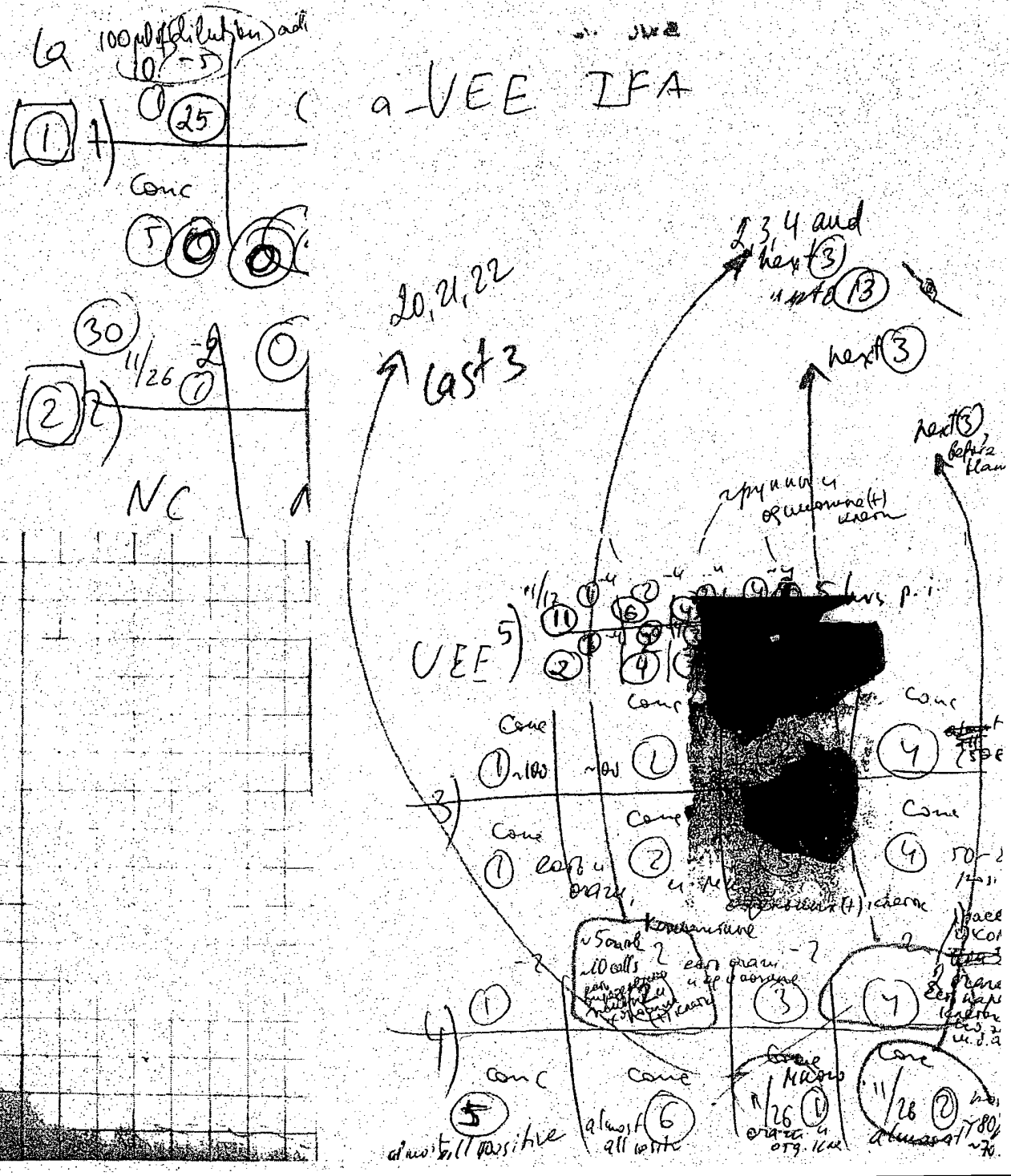


12/4/94 TEMPLATES FOR IN VITRO TRANSCRIPTION

[redacted]

① P8-1/NotI 1μl (out of 12) pV300α KpnI
② P10-1/NotI 1μl (out of 12) pV3014Δ RsrII-SpeI
③ pV300α/NotI 1μl (out of 12) full length

IFA [redacted] & VEE-Ag



12/5/1994

Plaque assay

1, 2	② transfection 11/30/94	$10^{-2} \div 10^{-7}$	~ 3 x 10 3	
3, 4	④ 11/30/94	- " -	7 x 10 2	
5, 6	⑤ 11/30/94	- " -	1 x 10 7 pfu/ml 1 x 10 7	
7, 8	⑥ 11/30/94	- " -	7 x 10 6	
9, 10	① transfection 11/26/94	$10^{-2} \div 10^{-7}$	0 plaques	
11, 12	② 11/26/94	$10^{-2} \div 10^{-7}$	1 plaque in 10^{-2} 100 ? or 0 ?	
13, 14	③ 11/26/94	$10^{-2} \div 10^{-7}$	0 plaques	
15, 16	② 11/30/94 new tube	$10^{-2} \div 10^{-7}$	7 x 10 3	
17, 18	④ 11/30/94 new	$10^{-2} \div 10^{-7}$	7 x 10 2	
19, 20	① 11/30/94 new	$10^{-2} \div 10^{-7}$	0 plaques	

IN VITRO TRANSCRIPTION

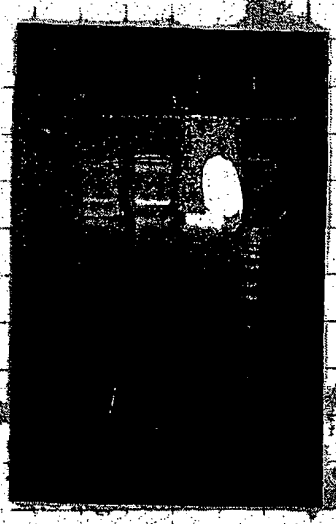
all as LSN in 11/14

P8-1 (Δ1404)
P10-1 (Δ3438)
P6-2 (Δ404)
pV300 except

DNA were added 1 µl (P6-2 - 1 µl) and 0.9 100mM GTP was added to pV300 and P6-2

12/6/94 Plasmid DNA

1) p2-20
2) CSN
3) 3526



TRANSCRIPTION

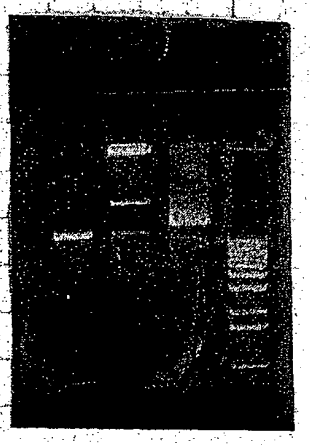
1) CSN
2) CSN
3) P5-1
4) V3000
5)

	CSN	P5-1	V 3000
water	9.5	5.4	9.3
5x buf	8	8	8
25mM NTP/G	12	12	12
25mM cap	4.8	8.4	7.8
DTA/RNAT	0.5	1.5	0.5
100mM GTP	0.7	0.2	0.9
10x Buf	4	4	4

4 hrs 37°C

12/7/94 1) Checking transcription reaction

2) ClaI - cleavage of p2-20, CSN-plasmid, 3526



12/7/94 TRANSFECTIONS 16 hrs

1) 12/6 LSN + 12/6 PS-1 20+20

2) 12/6 LSN + 12/5 P10-1 20+20

3) 12/6 PS-1 20

4) 12/5 P10-1 20

5) 3014

6) NC (no RNA)

12/8/94 TRANSFECTION EFFICIENCY 17 hrs p.e.

a-LSN (17 hrs p.e.)

①	②	③
100%	100%	"0"
④	⑤	⑥
"0"	"0"	"0"

① and ② - practically, 100% of cells are LSN-positive (~80% of cells in culture)

③ & ④ - practically 100% negative, but there are few "positive" cell which probably come from ① and ②

⑤ and ⑥ - negative, few "positive" cells, as in ③ & ④, may come from positive ① and ②

a-VEE (17 hrs p.e.)

①	②	③
100%	100%	0 (?!)
④	⑤	⑥
0	100%	0

INFECTIVITY ANALYSIS OF MEDIA (infection in CalTEC slides w/100 µl of appropriate dil; 1 hr at 37°C + 300 µl warm med; analysis 13 hr

a-Ga

10^{-5} ①	10^{-5} ②	conc ③	conc ⑦
23	4	0	0
conc ④	conc ⑤	10^{-4} ①	10^{-4} ②
0	0	254	71

2.8×10^7 i.u./ml for LSN-Ag

0.9×10^7 i.u./ml — " —

a-VE

conc ① (some areas cleared and cells are further spindly)	conc ② (cleared to varying degrees and cells spindly)	conc ③ 100% (+)	conc ④ 10^{-4} have positive
10^{-5} ⑤	conc ⑥	10^{-7} ⑤	conc inc
100% (+)	0	some areas and cells (+)	0

about 100 i.u./ml for VEE-Ag.

PLAQUE ASSAY

1	①	$10^{-2} \div 10^{-4}$	large + small 4×10^3 pfu/ml
2	②	$10^{-2} \div 10^{-4}$	2×10^2 large, 7 small
3, 4	③	$10^{-2} \div 10^{-4}$ $10^{-5} \div 10^{-7}$	1×10^5 pfu/ml large
5	④	$10^{-2} \div 10^{-4}$	0
6	⑤	$10^{-6} \div 10^{-8}$	(2.5×10^9 pfu/ml large (per small))
7	⑥	$10^{-2} \div 10^{-4}$	0

The results suggest that LSN and PS-1 are contaminated with VEE (+Pe virus). However, no VEE synthesis could be detected in transfection efficiency determination (failure)

2) LSN P10-1 20+2

3) P5-1 20

4) P10-1 20

5) 3014

6) NC (no RNA)

a-VEE (18 hrs p.i.)

① 100%	② 100%	③ 0 ((?!)
④ 0	⑤ 100%	⑥ 0

(LSN-positive) cells in cinch

③ ④ - practically 100% negative, but there are few "positive" cell with probably come from ① and ⑤ and ⑥ - respectively few "positive" cells as in ③ ④, may come from positive ① and ②

INFECTIVITY ANALYSIS OF MEDIA (Infection on CaliTEK slides w/10 µl of appropriate dil; 1 hr at 37°C + 30 µl warm overlays)

a-Ca

10^{-5} ① 23	10^{-5} ② 9	conc ③ 0	conc ④ 0
conc ⑤ 0	conc ⑥ 0	10^{-4} ① 254	10^{-4} ② 97

2.8×10^7 i.u./µl for LSN-Ag
0.9×10^7 i.u./ml — " —

a-VE

① conc some areas and cells are positive	② conc about 10 percent	③ conc 100% (+)	④ conc 0 (-) even 5 hrs p.i.
10^{-5} ⑤ 100% (+)	conc ⑥ 0	10^{-7} ⑤ some areas and cells (+)	conc NC 0

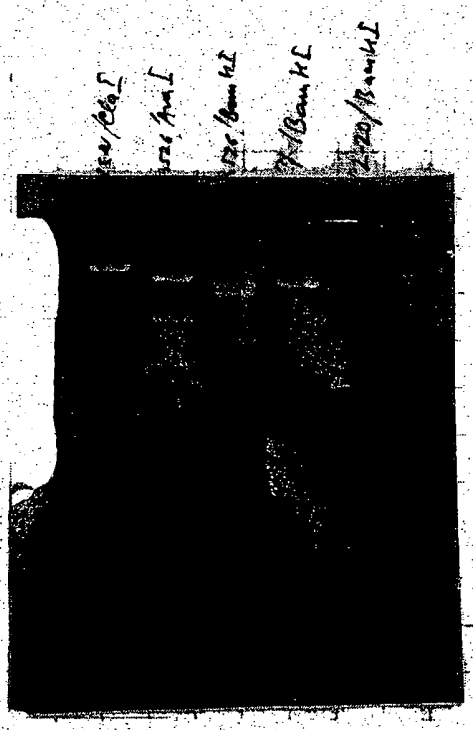
about 100 i.u./ml for VEE-Ag.

The results suggest that LSN P5-1 are contaminated with VEE virus. However, no VEE synthesis could be detected in transfection efficiency determination (above)

PLAQUE ASSAY

1	①	$10^{-2} \div 10^{-4}$	large + small 4×10^3 pfu/ml
	②	$10^{-2} \div 10^{-4}$	2×10^2 large, 7 small
3, 4	③	$10^{-2} \div 10^{-4}$ $10^{-5} \div 10^{-7}$	1×10^5 pfu/ml large
5	④	$10^{-2} \div 10^{-4}$	0
6	⑤	$10^{-6} \div 10^{-8}$	2.5×10^9 pfu/ml large (few small)
7	⑥	$10^{-2} \div 10^{-4}$	0
8	①	$10^{-2} \div 10^{-4}$	1×10^4 pfu/ml large
9	②	$10^{-2} \div 10^{-4}$	6×10^2 pfu/ml (small and larger)
10		NC	0

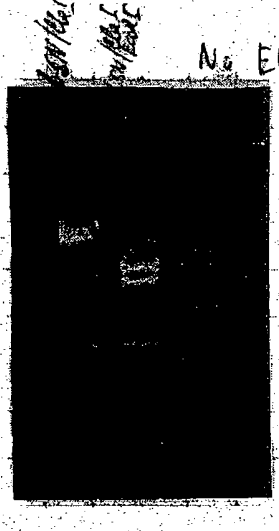
12/8/94



1) in both AvaI - and BamHI-cleaved 3.5 kb one additional band is present (why?)

2) p2-20 cleavage/BamHI looks strange

No EcoRI site in LSN gene!



12/9/94 P5-1; P6-2; LSN were transformed into DH5α,
12/10/94 single colonies grown in 3 ml of LB-Amp each,
12/11/94 mini-preps were done with fresh LSTET & STET
(dissolved in 50 µl water - sterile)

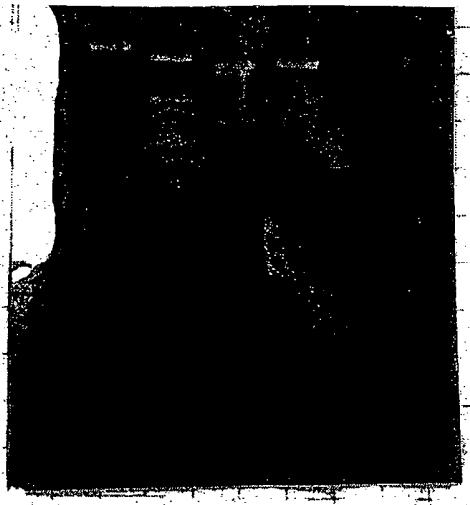
10 µl is cleaved o/n at 37°C w/NotI,
precautions were taken against contamination

12/12/94

(2x) new Phenol-chloroform
+20 µl NaAc 3M
+1000 µl EtOH (abs)
-70°C

TRANSCRIPTION

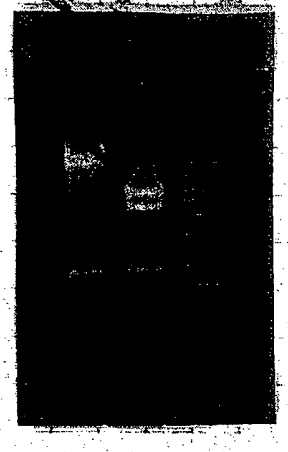
	lassa	P6-2	P5-1
water	8.5	8.5	5.4
5x	8	8	8
25mM NTP/G	12	12	12
25mM cap	4.8	4.8	8.4
DNA/NotI	1.5	1.5	1.5
100mM GTP	0.7	0.8	0.7
10mM	"	"	"



size one additional band is present (why?)

2) p2-20 cleavage/SamI looks strange

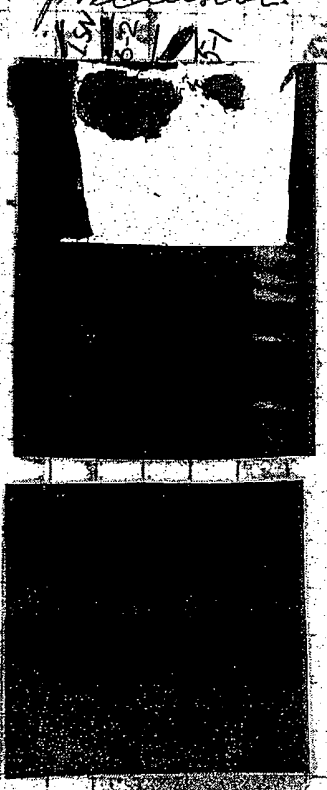
No ECOLI site in LSN gene!



A 134

12/9/94 P5-1; P6-2; LSN were transformed into DH5α
12/10/94 single colonies grown in 3 ml of LB-Amp each,
12/11/94 mini-preps were done with fresh LSET & STET
(dissolved in 50 µl water - stem 6)

10 µl is cleaved o/n at 37°C w/NotI,
precautions were taken against contamination



12/12/94

(2X) new Menst-chloroform
+ 20 µl NaAc
+ 1000 µl EtOH (abs)
- 70°C

TRANSCRIPTION

	Lassa	P6-2	P5-1
water	8.5	8.5	5.4
5X	8	8	8
25mM NTP/-G	12	12	12
25mM cap	4.8	4.8	8.4
DNA/NotI	1.5	1.5	1.5
100mM GTP	0.7	0.8	0.7
10x Buf	4	4	4

12/13/94

PLAQUE ASSAY CONTINUED

RT-PCR GibcoBRL Cat no. 18089-011 Superscript pre-amplification system

RNA was extracted from cell culture supernatant by guanidine isothiocyanate method (John's method)

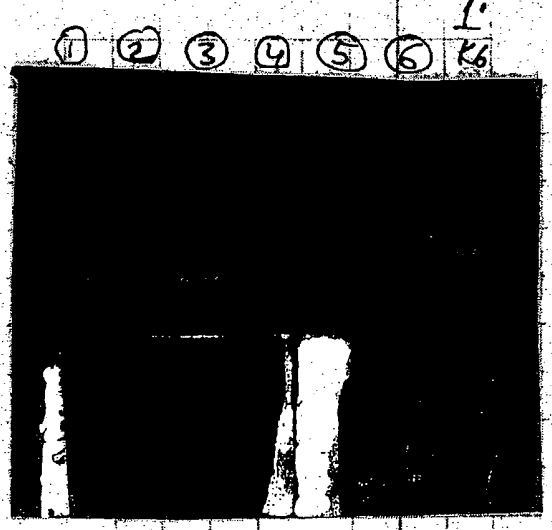
13 µl of RNA taken
+ 1 µl of random hexamers or 1 µl of 2 pM/µl of PVSI-R2
68°C 10 min ; 1 min on ice

+ 10x Synth buffer 2 µl
+ 10 mM dNTP mix 1 µl
0.1 M DTT 2 µl
SS II RT 1 µl
10 min Room Temp ; 42°C 50 min
15 min 68°C

+ 1 µl RNAse H
20 min at 37°C

+ 1.2 µl of VCR2
1.8 µl of VCF1
8 µl 10x Synth buf
67 µl water
AmpliTAQ 1 µl
+ mineral oil

1' at 94°C
and 30 cycles
1' at 94°C
2' at 52 °C
2' at 72 °C
30 cycles



Samples:

1. P1-5 } cDNA from random hexamers
2. P5-1 }
3. 3014
4. P1-5 } cDNA from E2 primer PVSI-R2 (Lorean)
5. P5-1 }
6. 3014

PCR fragments were done by "C" primers VCF1 and VCR2 (Korean)
expected size 860 bp

2/14/94 TRANSFECTION RNAs from 12/14/94 11:30

1) LSN + PS-2 13 + 18

2) LSN + PS-1 13 + 18

3) LSN

4) PS-1 18

5) P6-2

6) NC

First) time-course sample was taken 9 hrs p.e.
2nd) 21 hrs p.e
3rd)
4th)

IFA for determination of transfection efficiencies 21 hrs p.e
transf 12/14/94

LA

6 | 22 (Dr. Hruza) | 1

10^{-2} 9 hr p.e. | 10^{-2} 9 | 10^{-5} 21 | 10^{-5} 21 hr p.e. (dilution factor)

10^{-5} 24 hr p.e. | 10^{-5} 24 hr p.e. | 10^{-5} 27 hr p.e. | 10^{-5} 27 hr p.e.

2 | 84 | 6 | 63 (no. of positive cells)

100% | 100% | few positive, may be artifact | 0 | 0

VEE

80-90% VEE-pos (1) conc 27 — many positive single cells but only few areas

(2) conc 27 | (3) conc 27 | (4) conc 27

(5) conc 27 few areas or none!

nc | nc | nc

LA

20 | many | 0 | 86

(1) 27 conc | (2) 10^{-4} 27 conc | (1) 10^{-5} 29 conc | (2) 10^{-5} 27

(3) 27 | (4) 10^{-4} 27 | (5) 10^{-5} 27 | nc

0 | 0 | 0 | 0

although there are few positive cells which

Cells about
There are viru

a-Lassa: 1)	LSN P6-2
2)	LSN + PS-1
3)	LSN
4)	P6-1
5)	P6-2 <10
a-VEE: 1)	
2)	
3)	<10
4)	<10
5)	<10

RNA analysis by RT-PCR see next page

Plaque assay:

1)	plates (1) 2
2)	(2)
3)	(3)
4)	(4)

infection ... 1/100 ml ... monolayer ... lucite ... hr at ... 27 hr ... agar

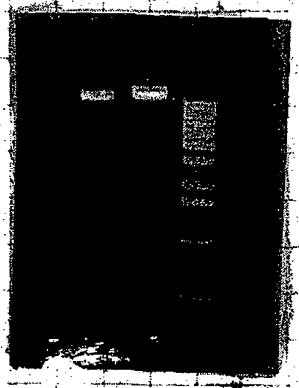
Ls (Ab) ... reaction ... (reading ...

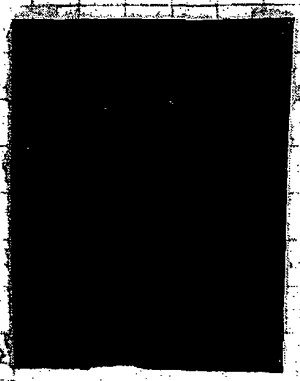
dilution factor ... lagoon 0.2 ... 10 ... 2 dilu ... cell (h ... virus ... (10 ...

"Sewage" 3526 Aqua I Rsrt II
3014 Swa I Rsrt II

12/15/94

(for synthesis)



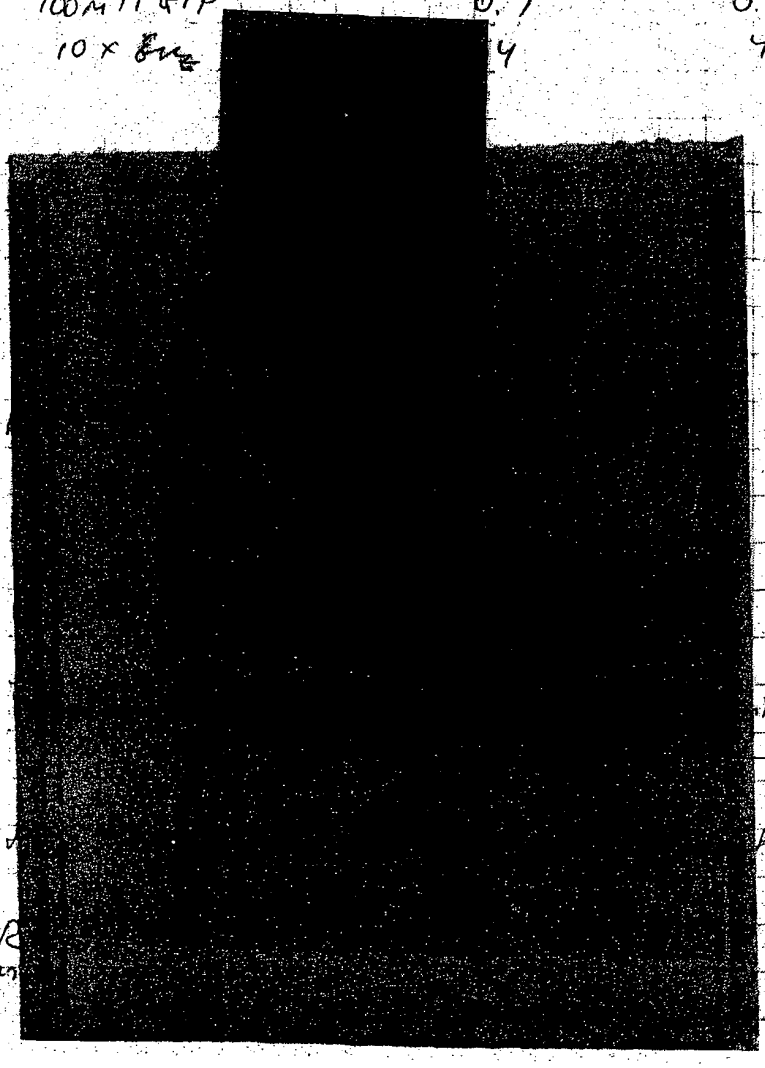


SM

TRANSCRIPTIONS

	P8-1(Sepa)	P10-1(Spl Rsr)	LSN
water	8.8	8.8	8.15
5×	8	8	8
25 mM NTP/-G	12	12	12
25 mM cap	4.8	4.8	4.8
~~DNA~~/NoFE	1.2	1.2	1.5
100 mM GTP	0.7	0.7	0.7
10× Enz	4	4	4

4 hrs at 37°C



12/16/94

1.
2.
3.
4
5.
6.
7.

+3 µl 10 mg/ml RNAse A
30' at RT

...ion manual (Jolins' Method)

12/17/94 1) RT-PCR as in protocol 12/13/94 compare to Gibco BRL protocol

2) TFA for VEE

3)

P11 3014 Δ RsrII SwaI 6µl + 2µl (E) + 2µl Cipp.

P12 35

4) TRANSFECTION:

Δ nsP2	1)	(SN)+	12
Δ RsrSpe	2)	SN +	
	3)	SN	
Δ nsP2	4)	P8-1	30
Δ vc	5)	P10-1	

INFECTIVITY (24hr p.i.)

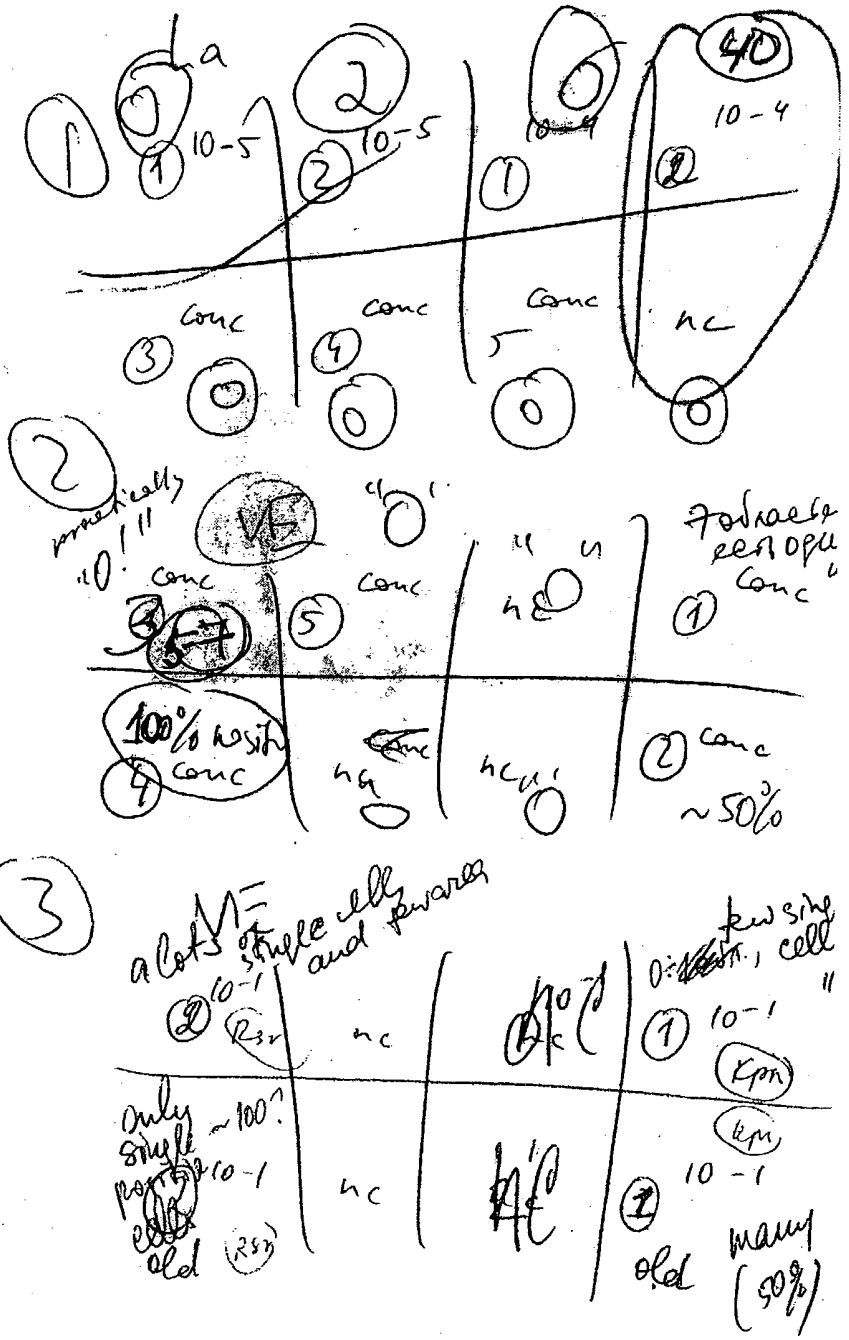
19hr p.i.	1)	1.4
	2)	
	3)	
	4)	
	5)	

RT-PCR

Plaque assay

1) Make (6) and (1)

2) (4) and (1)



12/18/94 1) TRANSFORMATION P11 & P12
2) IFA - LSN-Ag and VEE-Ag (transfection efficiency from 12/17/94)
3) RT-PCR gel (data on page 103)
4) INFECTIVITY ANALYSIS of transfection 12/17 & (12/14 - VEE)

A 140

12/19/94
1) Bulk, spotted
2) Plaque analysis of transformations 12/14 & 12/17
3) IFA - infectivity analysis of 12/17 transfection - frozen at -20 after acetone
4) RNA extraction from 12/14 transfections
5) P12 - plasmids inoculated into LB—Amp

4) 100 µl of PBS + 1 µl of medium containing particles sample or full control RNA from SScript kit
6 - with RNAse + without RNAse

5) Ligation P11 & P12 (again)

12/20/94 1) Plaque assay and IFA - counting
2) RT-PCR
3) Mini-preps P12 1÷16, cleavage w/ SmaI
4) Transformation?
5) Transcription?
6) 3014 Reverse ? and others?

7) TRANSCRIPTION

	P10-1	LSN	
Water	8.9	8.9	17.8
5x	8	8	16
25 mM NTP-G	12	12	24
2.5 mM cap	4.8	4.8	9.6
DNA/Not I	1.5	1.5	3
100 mM GTP	0.8	0.8	1.6
10 x Enz	4	4	8

[redacted]

3) [redacted]

all P12 1:16 are 0

P12-1 is 3526 bp T

2) Plaque analysis of transformations 12/14 & 12/17
3) IFA - infectivity analysis of 12/17 transfection - frozen at after acc
4) RNA extraction from 12/[redacted] transfections
5) P12 - plasmids inoculated into LB—Amp

4) 100 µl of PBS + 1 µl of medium containing particles sample or full control RNA from SScript
6 - with RNAse 7 without RNAse

6) Ligation P11 & P12 (early) **A 141**

12/20/94
1) Plaque assay and IFA - continue
2) RT-PCR
3) Mini-preps P12 1÷16, cleavage w/ SmaI
4) Transformation?
5) Transcription?
6) 3014 & Rev5wa [redacted] ? and others?

5) TRANSCRIPTION

	P10-1	LSN	
Water	8.9	8.9	17.8
5×	8	8	16
25 mM NTP-G	12	12	24
2.5 mM cap	4.8	4.8	9.6
DW A/ Not B	1.5	1.5	3
100 mM GTP	0.8	0.8	1.6
10× Buf	4	4	8

[redacted]

3)

all P12 1÷16 are

P12-1 is 352 Δ

[redacted]

12/21/94 1) TRANSFORMATIONS:

1) P2-2 272.5 Δ KpnI
2) P6-3 3014 Δ KpnI
3) P8-2 3000 Δ KpnI
4) P10-2 3014 Δ EcoRI SpeI
5) LSN - Replicon

2) RNA extraction (samples 12/17/94)

3 times plaque freeze/thaw

1-8 (exp ⑦) ate treated 30 min at RT with 50% of EWBR

1. ① from sup's of transfections 12/14/94 1 µl + 100 µl PBS
2. ⑫ — " — " — " —
3. ③ — " — " — " —
4. ④ — " — " — " —

not treated with EWBR

5. ⑤ —
6. ⑥ 100 µl of PBS + 0.5 µl of 1:10 diluted control
7. ⑦ — " — " — " —
8. 100 µl of PBS (- control)

3) TRANSFECTIONS 1900 Efficiency (16 hrs p.e.)

			LA		VE
1)	LSN + P2-1	10 + 18	90-100%	Some of the cells (groups) are brighter than the rest. Are these not transfected with helper? Many to, wt.	0% positive cells GFP
2)	LSN + P8-1	10 + 18	90-100%		20-30%
3)	LSN + P10-1	10 + 18	90-100%		50-60%
4)	LSN	10	90-100%		0
5)	P2-1	18	0		0
6)	P8-1	18	0		0
7)	P10-1	18	0		0

4) Plaques from P8-1 (12/17) taken (3 plaques, (+) medium frozen at -70°C)

INFECTIVITY ANALYSIS 23:30 infection (sup's taken 24 hrs p.i.)

	(24 hrs p.i.)	LA (15 hrs p.i.)	VE (15 hrs p.i.)	24 hrs p.i.
1)	LSN + P2-1	<10	<10 i.u/ml	<10 0/1 small bright area 0/10
2)	LSN + P8-1	2.5×10^4	1.7×10^4 i.u./ml	2 large 2 small areas (clusters of cells) 2 single cells (few) 30 areas/ml
3)	LSN + P10-1	$\sim 5 \times 10^7$	nt i.u/ml	single cells numerous only small cells, ICA
4)	LSN	<10	60 i.u/ml (!?)	<10 <10/ml clear area numerous
5)	P2-1	<10	<10 i.u/ml	<10 <10/ml — " —
6)	P8-1	<10; many dead cells !	<10 i.u/ml	<10 <10/ml — " —
7)	P10-1	<10	nt	<10 <10/ml — " —

Plaque analysis

5) LSN - Replicon

2) RNA extraction (samples 12/17/94)

1 ÷ 8 (exp 7) ate treated 30 min at RT with soup extract

1. ① from sup's of transfections 12/17/94 1µl + 100µl PBS
2. ②
3. ③
4. ④
5. ⑤

not treated with extract

6. 100µl of PBS + 0.5µl of 1:10 diluted control RNA
7. ⑦
8. 100µl of PBS (- control)

3 times pump treatment

3) TRANSFECTIONS 1900 Efficiency (16 hrs p.e.)

			LA		VE
1)	LSN + P2-1	10 + 18	90-100%	Some of the cells (groups) are brighter than the rest. Are these not transfected with helper? Many do, not	0%
2)	LSN + P8-1	10 + 18	90-100%		20-30%
3)	LSN + P10-1	10 + 18	90-100%		50-60%
4)	LSN	10	90-100%		0
5)	P2-1	18	0		0
6)	P8-1	18	0		0
7)	P10-1	18	0		0

4) Plaques from P8-1 (12/17) taken (3 plaques, (+) medium frozen at -70°C)

INFECTIVITY ANALYSIS 23:30 supernatant (sup's taken 27 hrs p.e.)

	(24 hrs p.i.)	LA (15 hrs p.i.)	VE (15 hrs p.i.) 24 hrs p.i.
1) LSN + P2-1	<10	<10 iu/ml	<10 0% small bright areas
2) LSN + P8-1	2.5×10^4	1.7×10^4 i.u./ml	2 large 2 small areas (20% 20%)
3) LSN + P10-1	~ 5×10^7	nt i.u./ml	single cells, stem cells, neuro ep. glia, small cells
4) LSN	<10	60 iu/ml (!?)	<10 <10/ml
5) P2-1	<10	<10 iu/ml	<10 <10/ml
6) P8-1	<10; many dead cells !	<10 iu/ml	<10 <10/ml
7) P10-1	<10	nt	<10 <10/ml

Plaque analysis

1) undiluted (10^{-1}) ÷ 10^{-3}
2) undiluted (10^{-1}) ÷ 10^{-3}
3) undiluted (10^{-1}) ÷ 10^{-3}
4) undiluted (10^{-1}) ÷ 10^{-3}
7) 10^{-4} undiluted (10^{-2})

94 ...rs... Setup Listing ...ersion ...

...:08P 12-22-94

394 ...rs: Setup ...

SINGLE λ 01 Abs Read average = 01

SW 260.0 SW 260.0

SW 260.0

1	0.0059			
2 PO1	0.6316	1,042.14 µg/ml	(1 mg/ml)	179.6 µM
3 PO2	0.6667	1,100.06 µg/ml	(1.1 mg/ml)	201.9 µM

...5= 0, 6= 0, 7= 0 8= 0

...cases= 0)

MW: 5567.6

5 ' GTG TAC AAC GAA CGT GAG '3

wt 1923 wt 1940

OD x dil. factor x 33 = µg/ml

mg/ml / MW = (µM)

8 x 10^7 i.u./... from 12/...

12/23/94

1. Gel RTPCR - for structural "ca" gene (12/17 samples) data on p...
2. Interfer... analysis - IFA a-LSN & a-VEE
3. PCR primers; PCR for nsp genes???
4. Mini-preps (5 or 6); cleavage w/ NotI
5. Infection (12/21 samples) 19:00

12/24/94

1) IFAs (acetone → -20°C) ~~15~~ 24 hrs p.i.
2) Not/I cleavage - check(?), pool, precipitate → -70°C.

12/26/94

TRANSCRIPTIONS

LSN/Not old

P2-2/Not

P8-2/Not } new

P12-1/Not

12/26/94 TRANSFECTION 19:00

			Efficiency (120 hrs p.e.) CA	VE
1.	LSN + P12-1	10 + 19	90%-100%	10-25%
2.	LSN + P2-2	10 + 19	90%-100%	0 !?
3.	LSN + P8-2	10 + 19	90%	5-10%
4.	LSN	10	90%	0
5.	P12-1	19	0	0
6.	P2-2	19	0	0
7.	P8-2*	19	0	0

← given plaques 3 days after starting w/ supernat Ved

RCR: *Not template contain only 400 bp. fragment

Media were taken (1 ml of Nos 1÷7) 28 hrs p.e., incubation continued at 37°C, and samples were taken

INFECTION OF BHK cells (in duplicate sets) 19:00 (19 hrs p.i.)
with media collected either (28) hrs p.e. or (47) hrs p.e

		28 (a-CA)	47	28 (a-VE)	47 hrs p.e
1	LSN + P12-1	~~<10~~ <10^3	~~<10~~ <10^3 i.u./ml	10^4 pfu/ml	80%
2	LSN + P2-2	<10	<10	<10	<10
3	LSN + P8-2	340 i.u./ml (single cells 10%)	~10/ml, 30 pfu/dish ~10 clusters	60%	100%
4	LSN	<10	<10	10^4 pfu/ml aprepator	<10
5	P12-1	<10	<10	<10	<10
6	P2-2	<10	<10	<10	Some positive single cells (dead?) many positive spots (dead cells)
7	P8-2	<10	<10	<10	

Plaque assay. See on p. 111

Helpers with KpnI fragment deleted (ns2 region)

Construct	Transfection date	Transfection efficiency, by IFA, %		Hrs p.e.	Media collected, hrs. p.e.	INFECTIVITY by IFA,		Hrs. p.i.	PLAQUE ASSAY
		anti-L	anti-V			anti-L, i.u./ml	anti-V, i.u./ml & f.f.u./ml		
LSN	12/26/94	90	0	20	28	<10	<10	19	
					47	<10	<10		
LSN+P2-2		90-100	0		28 & 47	<10 &<10	<10 & <10		
P2-2		0	0		28 & 47	<10 & <10	<10 & <10		
LSN+P8-2		90	5-10		28	340 singles	60%		
					47	10^4 (few areas 10 cells each + singles)	100%		
P8-2		0	0		28 & 47	<10 & <10	<10 & <10		
LSN	12/21/94	90-100	0	16	27	<10	<10		
LSN+P8-1		90-100	20-30			1.7x10^4	30ffu/ml	15	
						2.5x10^4	100ffu/ml 100iu/ml	24	
P8-1		0	0			<10	<10	15	
						<10, many dead cells in undiluted smpl	<10	24	
LSN+P2-1		90-100	0			<10	<10 or 10 ffu/ml		
P2-1		0	0			<10	<10		
LSN	12/17/94	100	0	15	24	<10	<10		<10
LSN+P8-1		100	5-25			1.4x10^4	70ffu/ml+ single cells	19	7x10^2
P8-1		0	0			<10	100%		3x10^5
LSN	12/14/94	100	0	21	27	<10	<10		<10
LSN+P6-2		100	100			6x10^6	80-100%	19	10^5
P6-2		0	0			<10	<10		<10
LSN+P6-2	11/30/94	100	70-80	22	27	4.5x10^6	2000ffu/ml	27	7x10^2
P6-2		0	0			<10	100%(undil)		7x10^6
	CEF								
LSN+P6-2	11/26/94	5	0	?	?	?	80%(undil)		0 - 10^2
P6-2		0	0	?	?	?	nt		0
LSN+P2-1	10/24/94	60-70	<1	22	?	?	?		0
LSN+P2-1	10/13/94	?	?	?	?	<10^3	<10^3		?
SN	10/1/94	60-80	0	18	?	nt	nt		nt
SN+P2-1		60-80	0			<10^4	<10^4		nt
P2-1		0	0			nt	nt		nt

ΔXpnI helpers

Construct	Date of transfection	Efficiency of transfection LA	UE	p.e., hrs	Media collected, hrs	IFA (14/wi) LA	IFA (14/wi) UE	His p.i.	Plaque pfu/ml
(2-2)	12/26	90%	0%	20	28 and 47				
(8-2?)		90%	0						
2-2		90%	5-10%						
8-2		0	0						
		0	0						
SN	12/21/94	90-100%		16	27	<10	<10		<10
3-1	12/21/94	90-100%	20-30%	16	27	1.7×10^4/15 hrs p.i.	30 areas/ml/15 hrs p.i.		
						2.5×10^4/24 hrs p.i.	160 areas/ml/24 hrs p.i.		10^2–10^3 pfu/ml
							~5 single cells ~10^2/ml		
8-1	12/21/94			16	27	<10/15 hrs p.i. many dead cells; <10/24 hrs p.i.	<10/75 hrs p.i. <10/24 hrs p.i.		240
SN	12/17/94	100%	0	15	24	<10	<10		<10
1	12/17/94	100%	5-25%	15	24	1.4×10^4/19 hrs p.i.	70 areas/ml + single cells/19 hrs p.i.		7×10^2
2-1	12/17/94			15	24	<10	100% of cells are positive		3×10^3
SN*	12/14/94	100	0	21	27	<10	<10		<10
2-2*	12/14/94	100%	100%	21	27	6×10^6/27 hrs	80–100% positive/19 hrs		10^3
2*	12/14/94			21	27	<10	<10		<10
						PS+ gives 8×10^7 i4/ml !!			
2-2	11/30/94	100%	70-80%	22	27	$>10^6$/27 hrs	2×10^3 areas/ml		7×10^7
2-2	11/30/94			22	27	<10	all positive (undilut)		7×10^6
6-2	11/26/94	5%	0	?	?	?	80% positive (undil)		0
6-2	CHICK	0	0	?	?	?	wt		0
SN	12/21/94	90%-100%	0	16	27	<10	<10		
2-1	-"-	90-100%	0	16	27	<10	<10		
2-1	-"-	0	0	16	27	<10	<10		
P2-1	10/24/94	60-70%	<1	22					0
P2-1	10/13/94	?	?			<100 i4/ml	<100 areas/ml		?
N		60-80%	0?	18		wt	wt		wt
+ P2-1	10/1/94	60-80%		18		<10	<10		wt
P2-1		0	0? high background	18	?	wt	wt		wt

110

1/95 DNA PCR

①	LSN template	new	(18 primers 10 µM) 40 °C annealing	0.1 µM DNA
②	P8-2 template	—	(— 10 µM)	— " —
③	LSN template	—	(— 200 µM)	— " —
④	old P8-2 (PCR grade)		(— 10 µM)	0.5 µM DNA
⑤	-11		50 °C annealing	
⑥	-2			
⑦	-3			
⑧	-4			

1 P8-2 (
2 P8-2 (
3
4

1/8/95

TRANSFECTION

12:00

1. LSN + P2-2
2. P2-2
3. LSN + P6-3
4. P6-3
5. LSN + P8-2
6. P8-2
7. Cells electroporated
8. cells not electroporated

18:00

IFA w/samples collected (undiluted)

	2 hrs p.e.	50 hrs p.e.		
1. LSN + P2-2	<10	<10	<10	<10
2. P2-2	<10	<10	<10	<10
3. LSN + P6-3	~1x10⁵/ml	100% cracks?	100%	100%
4. P6-3	<10	<10	<10	<10
5. LSN + P8-2	~6x10³/ml	~5-6x10³/ml	100%	100
6. P8-2	<10	<10	<10	<10

18:00

Plaque assay n⁻² ÷ n pfu/ml (undiluted sups + diluent & 100 µl per well)

Samples: 2 & 50 hrs p.e

1.	① 2 hrs p.e.	10⁻² ÷ 10⁻⁴	< 100	13	① LSN-P2-1	< 10
2.	② 2 hrs p.e		< 100	14	② LSN-P2-2	< 100
3.	③ 27 hrs p.e		5 x 10³ (0.2-3 mm)	15	③ LSN-P8-2	6 x 10³ (0.2-1 mm)
4.	④ 27 hrs p.e		< 100	16	⑤ P2-1	< 10
5.	⑤ 27 hrs pe		1 x 10³ (0.2-1 mm)	17	⑥ P2-2	< 10
6.	⑥ 27 hrs pe		< 100	18	⑦ P8-2	< 100
7.	⑦ 50 hrs pe		< 100			
8.	⑧ 50 hrs pe		< 100			
9.	⑨ 50 hrs pe		~1.5 x 10⁵ (0.2-3 mm)			

1 x 10³ (0.2-1 mm)

28 hrs p.e.; 10⁻² ÷ 10⁻⁴ dil

65

TRANSFECTION 12/2

6. P8-2
7. Cells electroporated
8. cells not electroporated

18:00

IFA w/samples collected 27 & 50 hrs p.e.
(undiluted)

	27 hrs p.e.	50 hrs p.e.		
1. LSN + P2-2	<10	<10	≤10	≤10
2. P2-2	<10	<10	≤10	≤10
3. LSN + P6-3	~1x10⁵/ml	100% cracked? non-adherent	100%	100%
4. P6-3	<10	<10	≤10	≤10
5. LSN + P8-2	~6x10³/ml	~5-6x10³/ml	100%	100
6. P8-2	<10	<10	≤10	≤10

Maybe reasonable numbers already exist
many cells
pfu/ml
Recombinants just ar
Mean
Mean
cells
Infected
Electropo
Plaque assay
Samples or from

A 150

18:00

Plaque assay $n^{-2} \div n^{-4}$ pfu/ml (undiluted Sup-s + diluent & 100 µl per well)

Samples 27 & 50 hrs p.e.

1.	① 27 hrs p.e.	$10^{-2} \div 10^{-4}$	<100	13.	① LSN P12-1	<100
2.	② 27 hrs p.e		<100	14.	② LSN P2-2	<100
3.	③ 27 hrs p.e		5×10^3 (0.2-3 Ø mm)	15.	③ LSN P8-2	6×10^3 (0.2-1 Ø mm
4.	④ 27 hrs p.e		<100	16.	④ P12-1	<100
5.	⑤ 27 hrs pe		1×10^3 (0.2-1 Ø mm)	17.	⑥ P2-2	<100
6.	⑥ 27 hrs pe		<100	18.	⑦ P8-2	<100
7.	① 50 hrs pe		<100		28hrs p.e.;	$10^{-2} \div 10^{-4}$
8.	② 50 hrs pe		<100			
9.	③ 50 hrs pe		~1.5×10^6 (0.2-3 Ø mm)			
10.	④ 50 hrs pe		<100			
11.	⑤ 50 hrs pe					
12.	⑥ 50 hrs pe		~2.5×10^6 (0.2-2 Ø mm)			

1×10^3 (0.2-1 Ø mm)

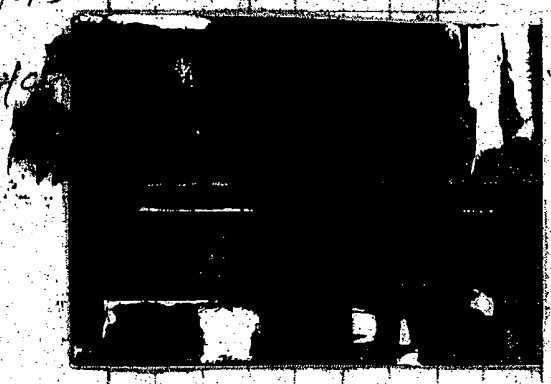
TRANSFECTION

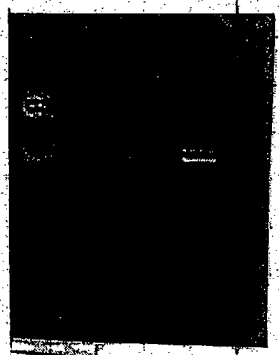
Cleavage 3014 1) Aat II
2) Kpn I

P13	3014 Δ Aat II	5 µl + 11 µl water + 2 µl ⓒ + 2 µl Cl	
P14	3014 Δ Aat II T4	5 µl + 11 µl water +	
P15	3014 Δ Kpn I T4	5 µl + 11 µl water +	
P16	3014 Δ Kpn I T4	1 µl + 15 µl water +	

1/13/95

1/18/95





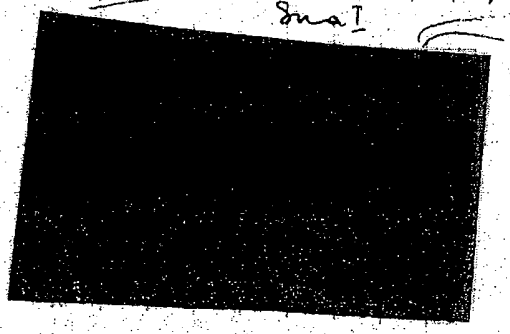
Further cleavage

Eco RI

P13 5
P14 5
P15 7; 8; 9; 11; 13

3014
P13 1÷5 1 P14 1÷4 1, 7 & 9 11 13 P15 3014

P15-7, 8, 9, 11, 13
Sma I
3014



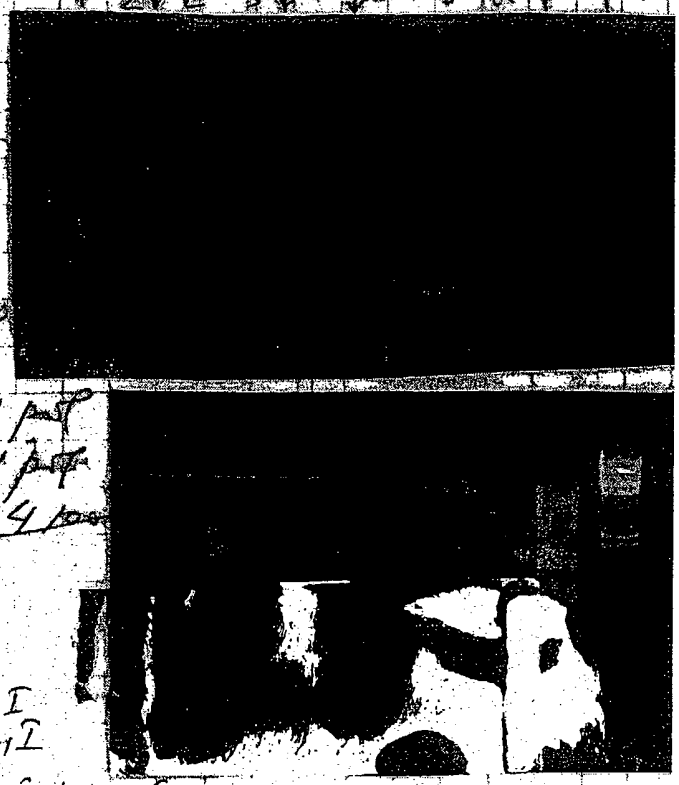
Cleavage

P13 1,2 } Aat II Rsr II
P14 2,3 }
P15 7,8,9 Kpn I Rsr II

P13-1: 3014 Δ Aat II
P13-2: 3014 Δ Aat II

P14-3: 3014 Δ Aat II/T4 pol

P15-7: 3014 Δ Kpn I/T4 pol
P15-8: 3014 Δ Kpn I/T4 pol
P15-9: 3014 Δ Kpn I/T4 pol



P13-2
P14-3 } Not I o/n ; Kpn I
P15-7 Aat I
Kpn I

1/19/95

TRANSCRIPTIONS

CSN
P13-2
P14-3
P15-7

Results of PLAQUE Assay (24 hrs post stain) 1/26/95

TRANSFECTION 12/26/94	1. ① - 28 hrs } CSN	$< 10^2$	1×10^3 (65 hrs post staining)	0.2 - 1 mm ∅	5×10^3
	2. ① - 47 hrs } P12-1	$< 10^2$	4×10^4 (0.2) - 1 mm		5×10^4
	3. ⑤ - 28 hrs } P12-1	$< 10^2$	$< 10^2$		$< 10^2$
	4. ⑤ - 47 hrs }	$< 10^2$	$< 10^2$		$< 10^2$
TRANSFECTION 1/4/95	5. ① CSN + P2-1	$< 10^2$	$< 10^2$		$< 10^2$
	6. ② CSN + P8-1	$< 10^2$	1×10^3	0.1 - 0.2 mm	5×10^3
				0.2 - 1 mm	

P 14-3 : 3014 Δ AatII/T4 pol

P 15-7 : 3014 Δ KpnI/T4 pol
P 15-8 : 3014 Δ KpnI/T4 pol
P 15-9 : 3014 Δ KpnI/T4 pol

A 153

P13-2
P14-3
P15-7
} Not I o/n ; KpaI AatII KpaI KpnI

1/19/95

TRANSCRIPTIONS

CSN
P13-2
P14-3
P15-7

Results of PLAQUE ASSAY (24 hrs post stain)

TRANSFECTION 12/26/94

1. ① -28 hrs } CSN + P12-1 $< 10^2$ — 1×10^3 (65 hrs post staining) 0.2 - 1 mm ∅
2. ① -47 hrs } CSN + P12-1 $< 10^2$ — 4×10^4 (0.2 - 1 mm)
3. ⑤ - 28 hrs } P12-1 $< 10^2$ — $< 10^2$
4. ⑤ - 47 hrs } P12-1 $< 10^2$ — $< 10^2$

TRANSFECTION 12/29/94

5. ① CSN + P2-1 $< 10^2$ — $< 10^2$
6. ② CSN + P8-1 $< 10^2$ — 1×10^3 0.1 - 0.2 mm
7. ③ CSN + P10-1 2×10^2 (0.1 - 1 mm) — 0.2-1 mm + agar (6 hrs) = ~ 1×10^3 total (65 hrs)
8. ⑤ P2-1 $< 10^2$ — $< 10^2$
9. ⑥ P8-1 $< 10^2$ — $< 10^2$
10. ⑦ P10-1 $< 10^2$ — $< 10^2$

21/1/94 TRANSFECTION 11 am

% confluency				a-LA	a-VE
<1	<1%	40%	1) LSN + P13-2	90-100%	80-90% looks l
<1	<1%	40%	2) LSN + P14-3	90-100%	80-90% ahead
<1	<1%	40%	3) LSN + P15-7	90-100%	40-50% and i cells.
3-5	60%	90%	4) P13-2	0%	0%
3-5	60%	90%	5) P14-3	0%	0%
1-5	60%	80-90%	6) P15-7	0%	0%
(72) hrs p.e.	(54) hrs p.e.	(22) hrs p.e.		22 hrs p.e.	22 hrs p.e.

MANY DEAD CELLS

ALMOST NO DEAD CELLS

Media Samples were taken 29 hrs p.e.
54 hrs p.e.
72 hrs p.e.

	LSN 29	LSN 54	LSN 72	VEE
1				90-100%
2	} цвет сливки (10^{-4} р)			90-100 %
3				90-100 %
4	nt			90-100 %
5	nt			90-100 %
6	NT			40-100 %

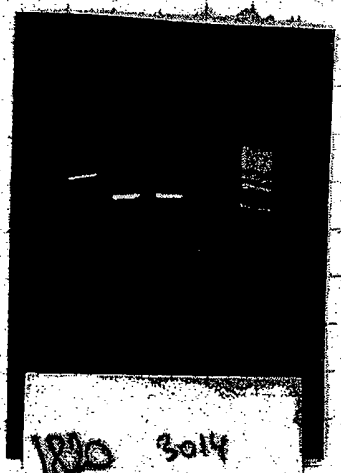
Plaque Assay

8/1/1995 $10^{-2} \div 10^{-4}$

1.	(8/1 - (1) - 27hr	LSN+P2-2	<100	<100	11.	8/1 - (4) - 50	P6-3	<100
2.	8/1 - (1) - 50hr	— " —	<100	<100	12.	— " — 78	— " —	<100
3.	8/1 - (1) - 78hr	— " —	<100	<100	(13.)	12/14/94 LSN P5-1		<100 Cut 2 spots in (1) 10^{-4} and 2 spots in (1) 10^{-3}
4.	8/1 - (3) - 27	LSN+P6-3	4×10^3 (0.1-1.5)	up to 1×10^4	14.	21/1 - (1) - 29 hr	LSN+P13-2	<100 in (1) 10^{-4}
5.	— " — 50	— " —	4×10^5 (0.1-3)	+ smalls	15.	— " — (2) — " —	LSN+P14-3	4×10^4 (1 mm)
6.	— " — 78	— " —	8×10^5 (0.1-2)	+ smalls	16.	— " — (3) — " —	LSN+P15-7	1×10^5 (0.5-1)
7.	8/1 - (5) - 27	LSN+P8-2	1×10^3 (0.1)	1×10^3 (+s?)	17.	— " — (4) — " —	P13-2	3×10^6
8.	— " — 50	— " —	$(5\times10^6$ (0.1-1) (2s)	+ smalls	18.	— " — (5) — " —	P14-3	$\sim 1\times10^7$
9.	— " — 78	— " —	5×10^6	+ smalls	19.	— " — (6) — " —	P15-7	$\sim 1\times10^7$
10.	8/1 - (4) - 27	P6-3	<100	<100	(20.)	12/14/94 LSN P5-1	undil. (10^{-1})	<10 or cl

1/24/95

RCR 87(1)3014(2) P13-2(3) P14-3 (4) P15-7 [all Not-cleaved transcription templates]
primers PØ1
PØ2



Purification of RNA from
(5) 1/21/95 (P14-3, passage 10⁷ pfu/ml)

medium cleared 10' at 1.800 rpm
1) 600 µl of medium + 600 µl phenol-chloroform
2) 600 µl of medium + 10 µl RNAse
3) 600 µl of medium + 10 µl RNAse } 30' at RT + 600 µl phenol chlo

2.5 vol. EtOH (RNA grade) 0.8 vol NaAc
2 µl Glycogen solution (Genius kit)

1/25/95

Purification of RNA

1. 12/14/94 PC-1 + LSPM 250 µl Hw
2. — / — + 250 + 10 µl RNAse 30' at RT
3. P14-3 1/21/95 250 + 250 + 10 µl RNAse
4. — / — 250 + 250 + 10 µl RNAse + 5 µl DNAse
5. P15-7 1/21/95 250 + 250 -
6. — / — 250 + 250 + 10 µl RNAse
7. medium 250 + 250 + 10 µl RNAse +
8. medium 250 + 250 + 10 µl RNAse + 5 µl DNAse

6

RNA isolation from growing media

1. 14-3 250 + 250 + (10) µl RNAse
2. 14-3 250 + 250 + (10) — " — (+5) µl DNAse } phenol
3. medium 250 + 250 + (10) — " —
4. (1) — " — — " — (10) — " — (+5)
5. water 500 + (10)
6. water 500 — no phenol
7. PIS-7 250 + 250 w RNAse w DNAse phenol

+ 200 µl water → (4)

Shaker over night

1 µl of Glycogen

1) infect ④ LABTEK - BHK cells with P5-1 & LSN particles 1/27/94
dilutions 10^{-1} ; 10^{-2} ; 10^{-4} ; 10^{-5}

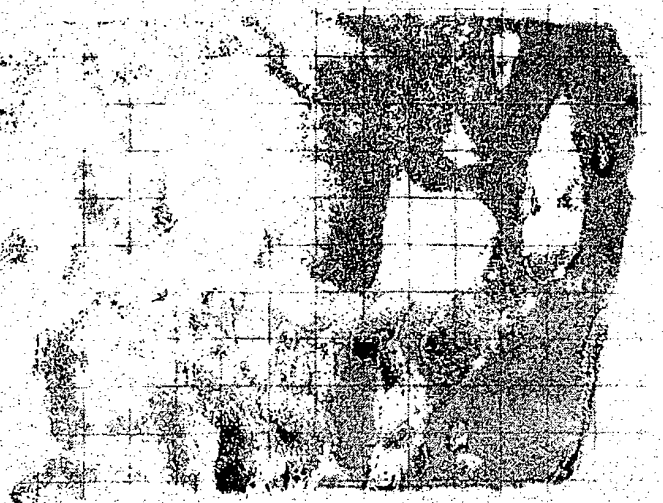
1) to a-VEE ab
2) to a-LSN conj
3) day 21 mouse ab (4) + NC
4) day 35 mouse ab + NC

infect t75 with P5-1 & LSN particles

2) Plaque assay

1.	P5-1	$10^{-2} \div 10^{-7}$		
2.	P5-1	$10^{-2} \div 10^{-7}$		on Sunday, next day after starting
3.	P5-1	$10^{-2} \div 10^{-7}$		
4.	P5-1	$10^{-3} \div 10^{-7}$	$10^3 \div 1\times10^4$? very small	
5.	P5-1	$10^{-3} \div 10^{-8}$		
6.	P5-1	$10^{-3} \div 10^{-8}$		
7.	P5-1	$10^{-3} \div 10^{-8}$		
8.	12/26 ③ 28	$10^{-2} \div 10^{-7}$	3 x 10 3	
9.	12/26 ④ 47	$10^{-2} \div 10^{-7}$	~ 1 x 10 7 - 10^8	
10.	~~12/26 LSN + P5-1~~	$10^{-2} \div 10^{-7}$	6 x 10 4	
11	12/17 P5-1	$10^{-2} \div 10^{-7}$	~ 1 x ~~10 7~~ - 10^8	
12		$10^{-2} \div 10^{-7}$	~ 1 x ~~10 7~~ ~ 10^8	

(65) hrs p.i.
0.5 x 10^3 L & ~4x
X
1 x 10^3 L & ~5x0
1 x 10^3 L & ~5x
2 x 10^3 L & ~5x
x 10^3 L & 5x
~~5~~ x 10^3 L & ~5x
5 x 10^3



1995 TRANSFECTION

		(12) hr monolayer	CPE*	unlabelled medium	labelled cells	labelled medium	by IFA, efficiency of transfection 16 hrs p.e (a-HA)	(a-LA)	(a-VE)
** 1.	P5-1 & LSV	~50÷60%	NO	+(taken)	+	−	0	100%	50÷70%
** 2.	P5-1 & HA	~50÷60%	NO	+	+	−	100	0%	50÷70%
** 3.	P12-3 & LSV	~50÷60%	NO	+	+	−	0	100%	50÷70
4.	LSV	~50÷60%	NO	−	−	−	0	100%	0
5.	HA	~50÷60%	NO	−	−	−	100	0%	0
* 6.	P5-1	~50÷60%	NO	−	−	−	0	0%	0
* 7.	P12-3	~50÷60%	NO	−	−	−	0	0%	0
8.	−	~50÷60%	NO	−	−	−	0	0	0

* floating cells

	(24) hr monolayer	CPE*	unlabeled medium (20 ca. Bahcax)	labelled cells	labelled medium
1.	~50-60-70%	no/begin?	+	+	+
2.	~50-60-70%	no/begin	+	+	+
3.	~50-60-70%	no/begin?	+	+	+
4.	same as above		−	−	−
5.	same	−	−	−	−
6.	same	−	−	−	−
7.	same	−	−	−	−
8.	same	−	−	−	−

	(33) hr monolayer	CPE*	unlabelled medium	labelled cell	labelled med
1	~40-50%	begin	+	+	+
2	~40-50	begin	+	+	+
3	~40-50	begin	+	+	+
4	~50	no/begin	+	+	+
5	~50	no/begin	+	+	+
6	~50-60	no	+	+	+
7	~50-60	no	+	+	+
8	~50-60	no	+	+	+

~~P20~~: 3014 : Δ Aat II Apa I

P19-1 : P5-1 Δ Tth111I SpeI (Δ capsid)

P21 : P5-1 Δ HpaI

DNA	1 µl
10 x BL	2
Water	15
Lig	2

P22 : P5-1 Δ HPaI - SpeI - T4 pol

P23 : 3014 Δ ApaI - AatII - T4 pol

TRANSCRIPTION HA and LSN

water	8.5	8.5
5 x buf	8	8
25 mM NTP-G	12	12
25 mM cap	4.8	~~4.8~~ 4.8
DNA/Not	2	2
100 mM GTP	0.7	~~0.8~~ 0.9
10 x Enz	4	4

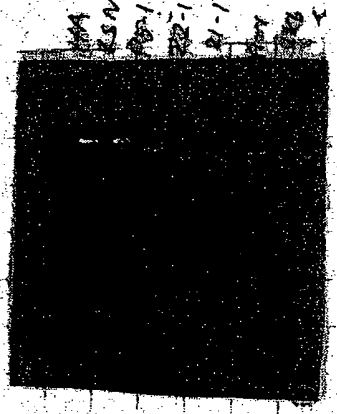
3/20/95

1. Transcriptions

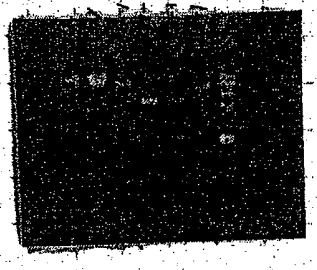
	HA	LA	5-1	21-1	22-1	19-1	
Water	~~20µl~~	~~20µl~~	~~20µl~~	~~30~~ ~~28~~	~~30~~ ~~28~~	~~38~~ ~~40~~	water
5x Buf	~~20~~	~~20~~	~~20~~	30	~~30~~		5x
25mM NTP/-G	(19) mix	(56) mix	(19) mix	(28) mix	(28) mix	(32) mix	NTP
25mM cap							cap
SpeA/NotI	1.5	4.5	2	4	4	5	
100mM GTP							GTP
10x Enz							Enz

8
12
4.8 (1.2 @ 100mM)
0.8

SpeA/NotI:



RNAs (1.5µl of each after 4 hr at 37°C)



3/24/95 TRANSCRIPTIONS

	LSN	HA	P5-1	P22-1 (P21-1)	P19-1
Water	4.4	7.4	2.9	1.8	1.7
5x	8	8	8	8	8
25mM NTP/-G	12	12	12	12	12
25mM cap	4.8	4.8	8.4	9.6	9.6
SpeA/NotI	3	3	4	4	4
100mM GTP	0.8	0.8	0.7	0.6 (0.5)	0.7
10x Enz	4	4	4	4	4
		1:1.5	1:3	1:4 (1:4.8)	1:3.4
		GTP:cap			

4/4/95 P23 : P5-1 : Spe I T4 after nucleocapsid → frame shift no GPS ORF

4/6/95 In vitro transcriptions

	HA	LSN	P5-1	P21-1
water	~~3~~ 3.5	7.4	2.9	0.9
5x buf	4	8	8	4
25mM NTP/-G	6	12	12	6
25mM cap	2.4	4.8	8.4	4.8
DNA/Not	2	4	4	2
100mM GTP	0.4	0.8	0.7	0.25
10x Enz	2	4	4	2.5
	20µl	40µl	40µl	20µl

4/9/95

	LSN	HA	5-1
water	~~9~~ 9	9.0 (9.0)	5
5x	8	8 (8)	8
25mM NTP/-G	12	12 (12)	12
25mM cap	4.8	4.8 (4.6)	8.4
DNA/Not I	1.5	1.5 (1.7)	2
100mM GTP	0.8	0.8 (0.8)	0.7
10x Enz	4	4 (4)	4

[redacted]

4/10/95

	P23-2	P21-1
water	5	2.8
5x	8	8
25mM NTP/-G	12	12
25mM cap	8.4	9.6
DNA/Not I	2 µl	3 µl
100mM GTP	0.7	0.6
10x Enz	4	4

[redacted] — LSN control

4/21/95 Oligonucleotides for GPs helper

CA1 0.14 o.u. × 100 × 33 μg/ml = 462 μg/ml
CA2 0.27 o.u. × 100 × 33 μg/ml = 891 μg/ml

CA1; 1 μl + 46.2 μl water = 10.0 μg/μl
CA2; 1 μl + 89.1 μl water = 10.0 μg/ml

Annealing mixture:
10 μl CA1; 10 μg/ml
10 μl CA2; 10 μg/ml
2.5 μl 5× Sequenase buffer
2.5 μl water
24.5 μl

~~65°C~~ 10 min anneal ~~to~~ 25°C slowly
65°C
42°C 10 min
37°C ~~10 min~~ 1 hr
Room temperature 1 hr

P24

vector	1 μl
fragment	1 μl
10× LigBuf	2
Ligase	2
Water	14

P24-1

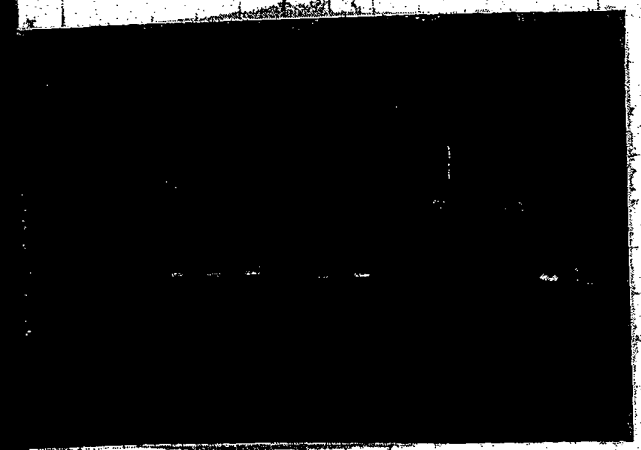
(X)		(1+1)		(1+5)	
vector	1	vector	1	vector	1
10×	2	fragm	1	fragm	5
Lig	2	10×	2	10×	2
Water	15	Lig	2	Lig	2
		Water	14	Water	10

P24 has annealed oligos CA1 and CA2 instead of P5-1
TthIIIE - Spe

/95 P24: colonies from ligations which contained cleaved TthIIII + Spe vector (cut, purified from gel) and 1/10 dilution of annealing mixture 4/21

A 163

from sixteen colonies 6 were positive:



purified with Wizard PCR prep kit

/95 Transfection BHK-80

1) LSN + 6-2
2. LSN + 10-1
3. LSN + 16-1
4. LSN + 23-1
5. LSN
6. 6-2 and 10-1
7. 16-1 and 23-1

	L	V-Rab	V-Mos	
1	90%	10-20	10-20	
2	90%	10-20	10-20	
3	60%	10-20	10-20	
4	90%	10-20	10-20	2
5	50% (less RNA)	0	1 +/-	
6	0%	0	0	
7	0%	0	0	

22 hrs p.e.

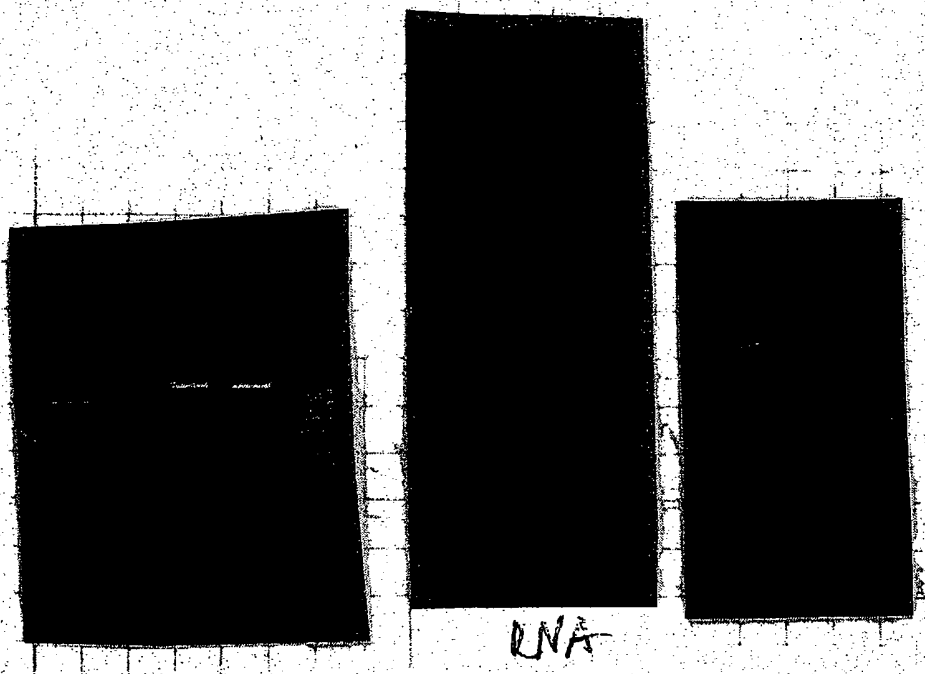
3 aceeum

(1) LSN - for transfection 5 1
(2) H4 — " — 6 —
(3) P5-1 — " — 7 —
(4) 23-2 (5-1 Spe TH) - for
5. 24-3 (5-1 + olig) for

cmeinp

1/28/95 TRANSCRIPTIONS

	LSN	HA	P5-1	P24-3	P21-1	P19
water	~~10.2~~	~~4.2~~	2.0	1.5	~~1.5~~	2.0
5X	~~8~~ 4	4	4	4	4	4
25mM NTP/G	~~12~~ 6	6	6	6	6	6
25mM cap	4.8	2.4	4.2	4.2	4.8	4.2
DNA/Not I	1.1	1	1.5	2	1.5	1.5
100mM GTP	~~0.8~~	0.4	0.35	0.35	0.25	0.3
10x Enz.	~~4~~	2	2	2	2	2



TRANSFECTIONS

1/ LSN + PS-1

2/ ~~LSN + HA + PS-1~~

1.	LSN + PS-1			1:1,000 and 1:10^{-5}
~~2.~~	~~HA + PS-1~~			
2.	LSN + P24-3	GPc	conc	
3.	LSN + (P21-1)	C	conc	
4.	LSN + P19-1	GPc	conc	
5.	LSN + P24-3 + (P21-1)	C+GPc	conc	and 1:1,000
6.	LSN		conc	
7.	PS-1 + P24-3 + [illegible]		conc	
8.	(P21-1) + P19-1		conc	

TRANSCRIPTIONS

	N-Rep	GP-Rep	HA-Rep	21-1	24-3
Water	4.9	4.7	4.2	4.9	5.9
5X	4	4	4	8	8
25 mM MP/G	6	6	6	12	12
25 mM cap	2.4	2.4	2.4	9.6	8.4
~~DNA~~ Not I	0.3	0.5	1	1	1
100 mM GTP	0.4	0.4	0.4	0.5 [illegible]	[illegible] 0.7
10 ~~Sag~~	2	2	2	4	4

	(21-1)	(24-3)
Water	5.4	6.4
5X	8	8
25 mM MP/G	12	12
25 mM cap	9.6	8.4
~~DNA~~ Not I	1	1
100 mM GTP	0.5	0.7
10 ~~Sag~~	4	4

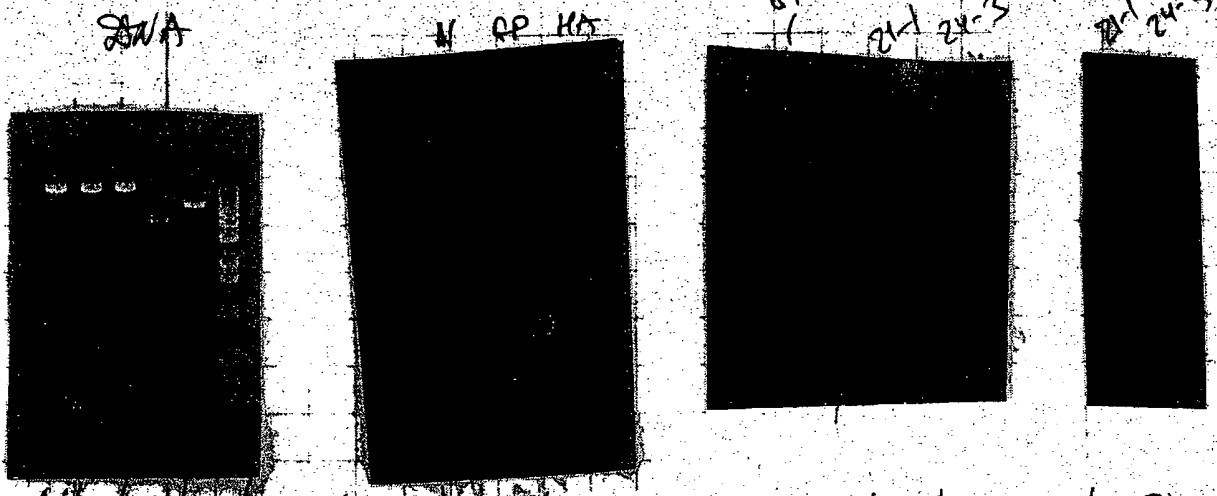
Water	2.5
5X	4
25 mM MP/G	6
25 mM cap	4.8
~~DNA~~ Not I	0.3
100 mM GTP	0.4
10 ~~Sag~~	2

·6

5/13/95 TRANSFECTIONS

1. N-Rep + 21-1 + 24-3
2. GP-Rep + 21-1 + 24-3
3. HA-Rep + 21-1 + 24-3
4. N
5. GP } -Rep
6. HA
7. 21-1 + 24-3

} ~ 10 µl each
couch-see gels

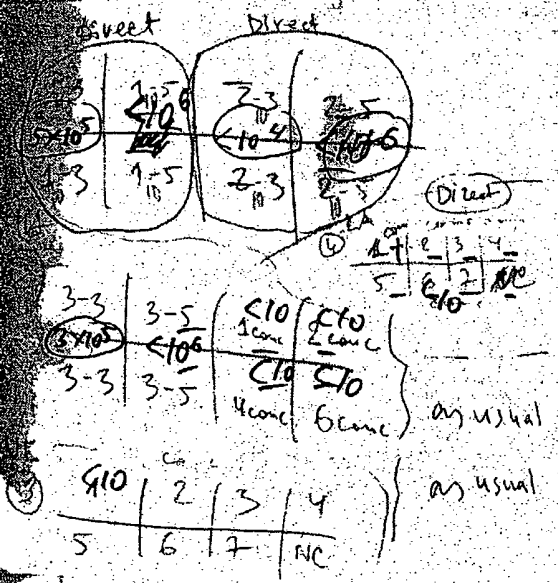


RNA

22 hr p.e. CSN VE HA CPE 24 hr p.

5/14/95	1	90%	70%	−	+/−
	2	pale 80-90% (??)	40%	−	+/−
	3	verypale 0-5%	70%	+	−
	4	50%	0	−	+/−
	5	pale 50% (??)	0	−	+/−
	6	0 !!!	0	+	−
	7	0 !!!	0	−	+/−

(2) (5) скучные клетки



Western

- a-L
- 1°° -a-VEE (NMAS)

Block: 100 ml PBS:
10% 10 g SKIM MILK
0.5% 0.5 ml Tween 20

Wash: 2 l PBS
0.5% 10 g SKIM M
0.025% 0.5 ml Tween 20

June 13, 1995

(1) Oligos synthesis for PCR mutagenesis
VEE capsid 1) H 141 → G-141 (cap H141) with Afl II site
2) universal capsid primer with Tth 111I site (capuniver)

- 3S4 Sequence Listing - (Version 1.02)

Name: CAPH141G to Proline 141 1:24:38P, 6/13/95

Total bases - 51
A- 8, G- 15, C- 10, T- 12, S- 0, S- 0, ?- 0, 9- 0
(Mixed bases- 0)

Seq: 1 GGCGC 5
AflII OK
5' CGC TTA AGC GCG GCC AGA ACG TCG TTG TCG ATC TTG CCT
TCC ACA GGC ATC GGC 3'

- 3S4 Sequence Listing - (Version 1.02)

Name: CAPUNIVERS 4:38:19P, 6/13/95

Total bases - 29
A- 15, G- 5, C- 1, T- 7, S- 0, S- 0, ?- 0, 9- 0
(Mixed bases- 0)

Seq: 1 GTGAC Tth 111I
5' AAA CAC ATA ATC TAC TCC GCC AAC ATA TTC T

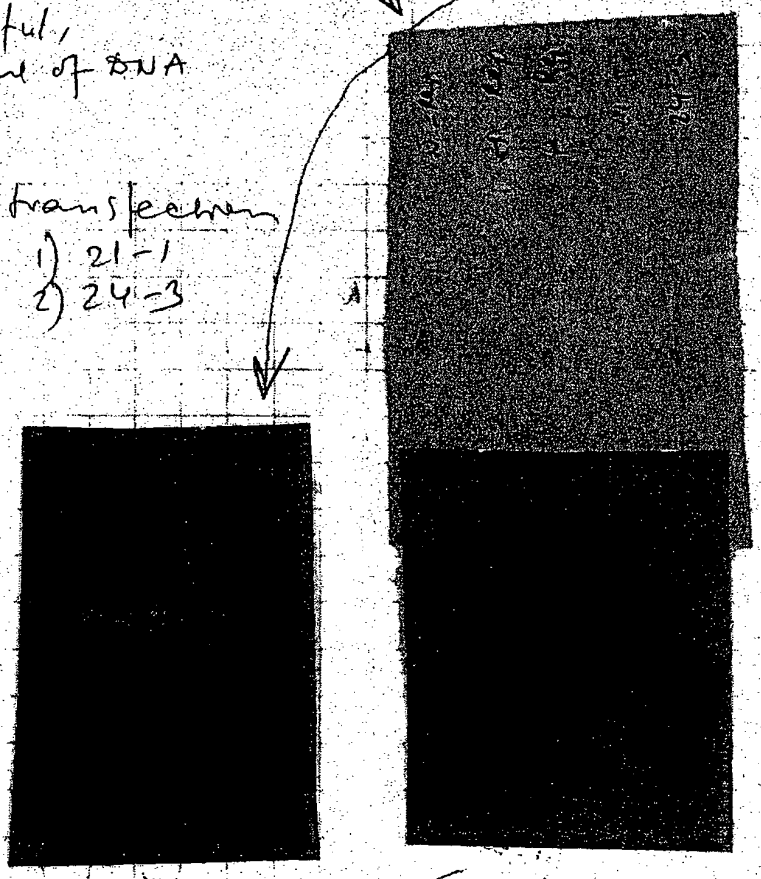
2.

② in vitro transcription

N-Rep, GR-Rep, HA-Rep } were made (p.125, in pencil)
should be mixed with
other preparation made as in (p.125, in pen)
mix and use for transfection

21-1 } unsuccessful,
24-3 } try 2μl of DNA

③ E. coli DH5α transfection
with 1) 21-1
2) 24-3



GTP cap 1:1.5 — mix! — GTP cap 1:3

June 14, 1995

① in vitro transcription

	21-1	24-3	
water	3.9	4.9	Note ?
5X	8	8	
25mM NTP/-G	12	12	
25mM cap	9.6	8.4	

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